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ORIGINAL PAPERS

SHORT COMMUNICATION

Acta Agronomica Hungarica, 56(1), pp. 1–10 (2008)
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EFFECT OF LOW LIGHT INTENSITY ON THE *VRN-H1* AND *VRN-H2* VERNALIZATION RESPONSE LOCI IN BARLEY (*Hordeum vulgare* L.)

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The flowering characteristics of a facultative × winter barley mapping population were evaluated in a series of controlled environmental tests in order to study the effect of low light intensity in association with various photoperiod regimes. Functional QTL analysis was used to determine the effect of low light intensity on the functioning of the *VRN-H1* and *VRN-H2* vernalization response genes and on the allele interactions. Low light intensity exerted the strongest modifying effect on these genes under a 12-hour photoperiod regime, which was intermediate between short and long daylengths. With this photoperiod more than 50% of the phenotypic variance in flowering was explained by the *VRN-H2* gene when high light intensity (340 μmol m^{-2} s^{-1}) was applied, but at low light intensity (170 μmol m^{-2} s^{-1}) the *VRN-H1* gene became the most important source of variation. There were also significant changes in the interaction between the alleles of the two *VRN-H* genes, implying that in addition to their role in vernalization-driven regulation, they may also participate in and be subjected to circadian-driven developmental regulation.

Key words: barley, flowering, light intensity, *VRN* genes

Introduction

Detailed studies on the effect of photoperiod on the flowering of cereals have established not only the various reaction types, but also the major genes regulating photoperiod sensitivity and their influence on flowering (Laurie et al., 1994; Karsai et al., 1997; Turner et al., 2005; Faure et al., 2007). In general, barley is a facultative long-day plant; its flowering is enhanced by a long photoperiod, but it is able to head under short photoperiods as well (Karsai et al., 2001; Laurie et al., 2004). Much less is known about the role and effect of light intensity in determining flowering in cereals. In spring wheat, Evtushenko and Chekurov (2004) examined the effect of low light intensity on flowering and found it to be mostly independent of photoperiod sensitivity and under the genetic control of a few recessive genes. They concluded that these recessive genes may coincide with the *eps* loci and that sensitivity to low light intensity may contribute a large portion of the flowering time variance between wheat cultivars of different geographical origin. In barley, it was found that light intensity, rather than being a major independent source of variance in flowering, played a chaperone role in modifying the effects of other environmental cues such as ambient temperature, photoperiod and daily fluctuating environmental factors (Karsai et al., 2007a; 2008). The application of low light intensity greatly accentuated the flowering time differences between various barley genotypes under conditions unfavourable for flowering. Thus, barley genotypes showing sensitivity to low light intensity could be separated from insensitive genotypes when low light intensity was accompanied by short photoperiod, supra-optimal temperatures during plant development or by synchronously applied factors that fluctuated daily (Karsai et al., 2007a; 2008).

Two major genes for vernalization response, *VRN-H1* and *VRN-H2*, have been identified in barley (Danyluk et al., 2003; Yan et al., 2003; von Zitzewitz et al., 2005). The candidate gene for *VRN-H1* is the MADS-box gene *HvBM5A,* which is the barley orthologue of the wheat *AP1* (*VRN1*) floral meristem identity gene. In genotypes with spring growth habit the *Vrn-H1* gene product can be detected early in plant development, resulting in early flowering, while in genotypes with winter growth habit the AP1 protein does not appear until the vernalization requirement has been met (Danyluk et al., 2003; von Zitzewitz et al., 2005). A region in the first intron of *HvBM5A* is thought to include the binding site for the repressor encoded by *VRN-H2* (Fu et al., 2005; von Zitzewitz et al., 2005). Deletion of this region leads to the dominant allele for spring growth habit. One or more of the three physically linked *ZCCT-H* genes on chromosome 4H, which are present in winter genotypes and deleted from facultative and spring genotypes, are candidate genes for *VRN-H2* (Yan et al., 2004; von Zitzewitz et al., 2005). Based on the flowering regulation model, vernalization saturation represses the activity of the dominant *VRN2* allele, allowing recessive alleles to be expressed at *VRN1*. This two-gene epistatic model has been demonstrated to be responsible for the vernalization requirement and thus for the growth habit in cereals (Yan et al., 2004; Dubcovsky et al., 2006; Kóti et al., 2006; Szűcs et al., 2007).

The aim of the present work was to study the effect of two major characteristics of light (light intensity and photoperiod) on flowering and on the *VRN-H1* and *VRN-H2* vernalization response genes in a barley doubled haploid population, which has been studied in detail. The availability of allele-specific primers for these genes made it possible to apply functional QTL analysis to measure the effect of each gene on the phenotype.

Materials and methods

Plant materials

The barley cultivars Dicktoo (D; facultative) and Kompolti Korai (K; winter) and the DH mapping population derived from the cross of D × K have been studied in depth at the genotypic and phenotypic levels (Karsai et al., 2005; 2006; 2007b; 2008; von Zitzewitz et al., 2005; Szűcs et al., 2006).

Phenotypic characterization

Controlled environment experiments were carried out in the phytotron facilities of the Agricultural Research Institute of the Hungarian Academy of Sciences, Martonvásár, Hungary using CONVIRON PGV type growth chambers (CONVIRON Ltd., Winnipeg, Canada). The technical parameters of the growth chambers, including light sources and control systems for temperature and light intensity, were detailed in Karsai et al. (2004). The effect of two light intensities (340 μmol m^{-2} s^{-1} and 170 μmol m^{-2} s^{-1}) in combination with three photoperiods (16 h, 12 h and 10 h) was examined in two separate experiments on two subsets of the D × K population. For the 340 μmol m^{-2} s^{-1} light intensity (referred to as high light intensity), the flowering time data of the 16, 12 and 10 h photoperiod treatments were taken from the photoperiod gradient experiments on 36 D × K lines published by Karsai et al. (2006). For the 170 μmol m^{-2} s^{-1} light intensity (referred to as low light intensity) a further 60 D × K lines were included in a separate experiment using the same photoperiods. All other conditions were the same in both experiments. The plant materials were vernalized for six weeks at 3°C with a 9 h light/15 h dark photoperiod regime at a light intensity of 10 μmol m^{-2} s^{-1}. The temperature was kept at a constant 18°C throughout the experiment. Each genotype was replicated twice within each treatment, giving an average plant density of approximately 60 plants m^{-2}. Genotypes that did not flower were assigned a flowering time of 150 days for statistical purposes.

Genotyping, linkage map construction and QTL analysis

The D × K linkage map consists of 236 loci of various types, with a total recombination length of 1107 cM and an average marker distance of 4.5 cM (Karsai et al., 2007b). The *VRN-H1* and *VRN-H2* loci were mapped with allele-specific primers in the D × K populations (von Zitzewitz et al., 2005; Karsai et al., 2005). Linkage maps were constructed using JoinMap 4.0 (van Ooijen, 2006). QTL analysis was performed using composite interval mapping (CIM) Model 6, with forward regression and backward elimination as implemented in WinQTL Cartographer v. 2.5 (Wang et al., 2007). Threshold levels were set using 500 permutations.

Results

The two parental lines differed from each other in their sensitivity to both photoperiod and light intensity (Fig. 1). Dicktoo was more sensitive to changes in these two environmental cues than Kompolti Korai. While low light intensity had no strong effect on flowering under a long photoperiod, it significantly delayed the flowering of both varieties under a 10-hour photoperiod, when Dicktoo was unable to flower.

The average flowering time of the two subpopulations did not differ from each other under photoperiod regimes of 16 hours (X_{high} = 51 days, X_{low} = 44 days; P = 0.061) and 12 hours (X_{high} = 77 days, X_{low} = 72 days; P = 0.121). At these photoperiods low light intensity accelerated flowering to some extent, but the effect was not significant. Under a 10-hour photoperiod low light intensity significantly delayed flowering (X_{high} = 87 days, X_{low} = 116 days; P = 0.000).

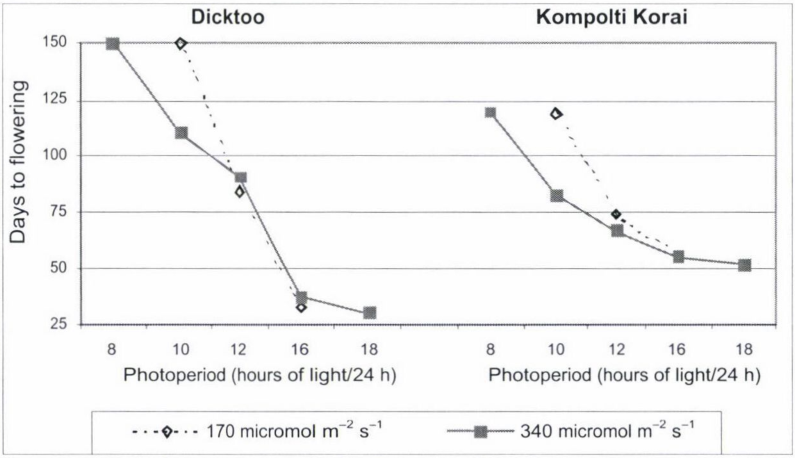

Fig. 1 .Flowering time characteristics of the two parental barley lines under various photoperiod regimes as a function of light intensity

When the role of the two *VRN* loci in flowering time was examined, it was found that both the photoperiod and the light intensity influenced their activity (Fig. 2). Under a long photoperiod (16 h) *VRN-H2* explained the largest portion of the phenotypic variance irrespective of the light intensity. The *VRN-H1* gene alone was only a significant though minor source of variance under high light intensity. The two genes together contributed more than 90% of the variance at both light intensities (R^2 high = 96.9%; R^2 low = 91.9%).

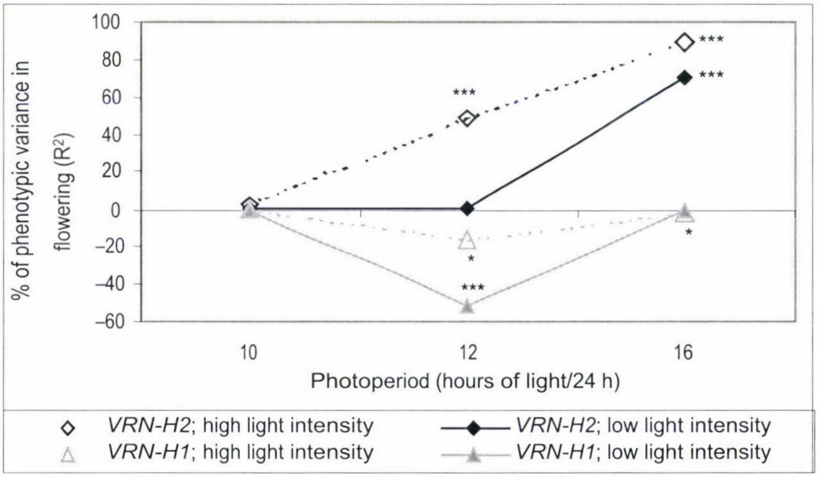

Fig. 2. Effect of photoperiod (10, 12, 16 h) and light intensity (high: 340 μmol $m^{-2} s^{-1}$, low: 170 μmol $m^{-2} s^{-1}$) on the role of the *VRN-H2* and *VRN-H1* genes in determining flowering, based on the portion of phenotypic variance explained in the Dicktoo × Kompolti Korai barley mapping population. (Data points on the plus portion of the vertical axis indicate that the allele with the larger added value was contributed by Kompolti, while values on the minus portion of the axis indicate that this allele was contributed by Dicktoo. *, **, ***: Significant at the P=0.05, 0.01 and 0.001 levels, respectively.)

Light intensity had the strongest effect on the *VRN-H* genes under the 12-hour photoperiod regime, which represents the borderline between long and short photoperiod regimes. While the effect of *VRN-H2* was highly significant under high light intensity, the activity of this gene could not be detected when low light intensity was applied. The effect of *VRN-H1*, on the other hand, was tripled at low light intensity, compared to high light intensity. More than 50% of the phenotypic variance in the flowering time under a 12-hour photoperiod was thus explained by *VRN-H2* under high light intensity and by *VRN-H1* under low light intensity. The bi-locus effect was highly significant at both light intensities (R^2 high = 78.8%; R^2 low = 52.8%). Under a short photoperiod (10 h) the two *VRN-H* genes did not influence the flowering time, irrespective of the light intensity applied.

As the bi-locus effects of the two *VRN-H* genes contributed the highest proportion of the phenotypic variance under 16- and 12-hour photoperiod regimes, the flowering characteristics of the four possible allele combinations were compared (Fig. 3). Under a long photoperiod the light intensity did not influence the type or degree of interaction between the allele phases of the two *VRN-H* genes. The Kompolti allele in *VRN-H2* (presence of the gene) resulted in later flowering irrespective of the light intensity level applied and this effect was not modified by the allele composition of the *VRN-H1* gene. The lack of the *VRN-H2* gene caused earlier flowering and made the effect of the allele composition of *VNR-H1* significant under both light intensities. Under a 12-hour photoperiod, however, the light intensity exerted a strong modifying effect on the interaction between the two *VRN-H* genes. At high light intensity the interaction between *VRN-H2* and *VRN-H1* was similar to that observed for the 16-hour photoperiod, except that the importance of the *VRN-H1* allele composition increased. At low light intensity level, the quantitative effect of the *VRN-H2* gene in repressing flowering diminished significantly. The presence or absence of the *VRN-H2* gene only influenced flowering when the Kompolti Korai allele was present in the *VRN-H1* gene. In the case of the Dicktoo *VRN-H1* allele, the *VRN-H2* gene had no apparent effect on flowering. When the effects of the two light intensities on flowering were compared under the 12-hour photoperiod regime, it became apparent that low light intensity only resulted in earlier flowering when the *VRN-H2* gene was present. In this case, however, its effect was mostly independent of the allele composition of *VRN-H1*.

Under the 10-hour photoperiod flowering was not influenced either by the individual effects of the *VRN-H* genes or by the various combinations of alleles.

Discussion

The results of the present experiments confirmed previous studies on the reaction types of barley varieties, showing that light intensity primarily influences plant development in association with other environmental stimuli (Karsai et al., 2007a; 2008). In *Arabidopsis* it was proven that changes in light intensity had a modifying effect on the period of the circadian rhythm (Zhou et

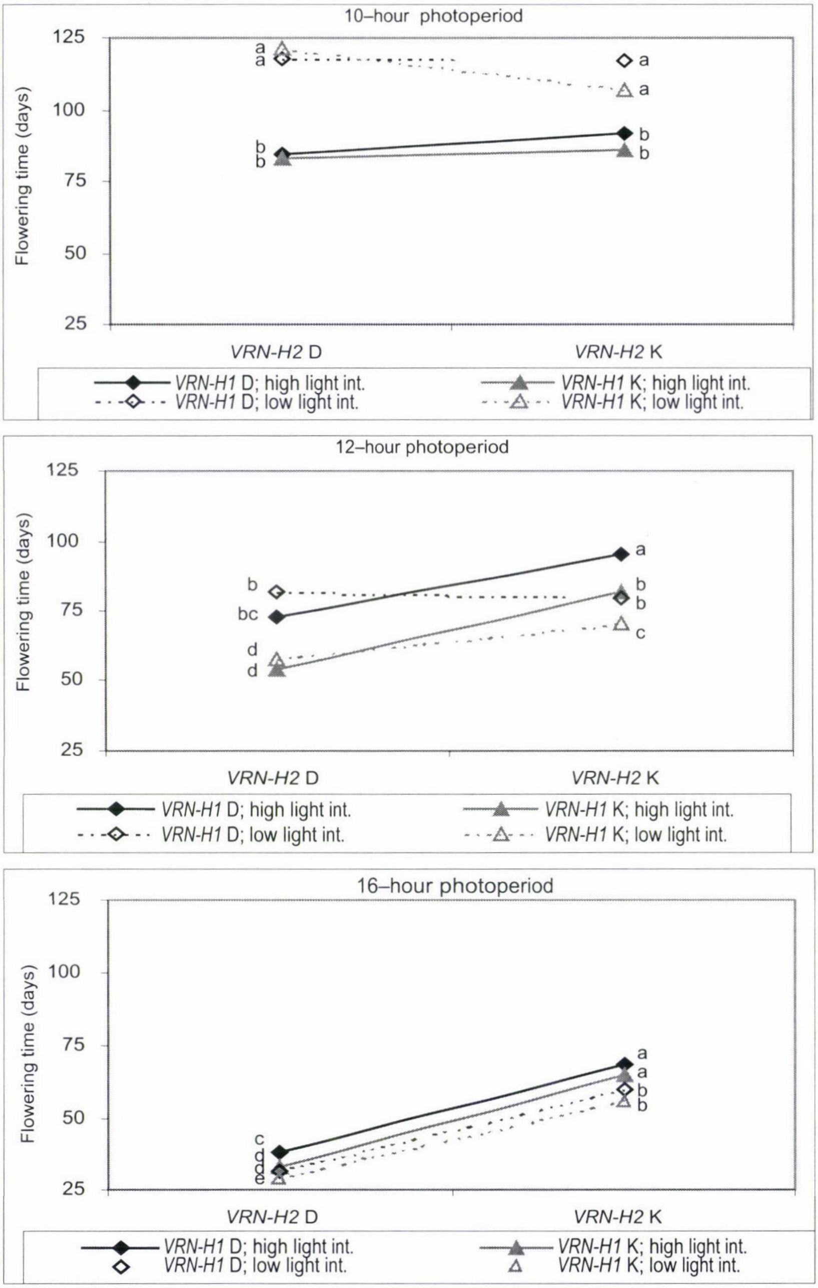

Fig. 3. Effects of photoperiod (10, 12, 16 h) and light intensity (high: 340 μmol m^{-2} s^{-1}, low: 170 μmol m^{-2} s^{-1}) on the association between the allele phases of the *VRN-H2* / *VRN-H1* genes in the Dicktoo (D) × Kompolti Korai (K) barley population, measured in terms of flowering time. (Within each photoperiod, data points labelled with the same letter were not significantly different from each other at the P=0.05 level.)

al., 2007). This phenomenon is thought to be part of the continuous adjustment of the period of the central oscillation throughout the light phase (Gardner et al., 2006). The relationship between light intensity and flowering, however, is not linear, as low light intensities combined with a change in the far-red/red ratio were found to participate in the shade avoidance mechanisms of plants living in natural habitats, resulting in earlier flowering (Vandenbussche et al., 2005). The connection between the shade avoidance mechanism of *Arabidopsis* and the sensitivity to low light intensity in cereals is not yet clear, but the flowering time characteristics of the facultative × winter barley population under long and intermediate photoperiods and the reaction types of spring barley varieties sensitive to low light intensity show strong similarity (Karsai et al., 2007a).

Functional QTL analysis on the two *VRN-H* genes underlined the fact that light intensity may play a significant role in modifying gene effects and relationships between the alleles. It has been established that the activity of the *VRN-H* genes in barley is influenced by photoperiods in addition to the primary role of low temperature vernalization (Danyluk et al., 2003; von Zitzewitz et al., 2005; Karsai et al., 2005; Dubcovsky et al., 2006; Trevaskis et al., 2006). The present work indicates that light intensity also exerts a significant effect on these genes, especially under photoperiod regimes intermediate between short and long day-lengths.

The barley *VRN-H2* gene, though different in structure, shows strong functional homology to the *Arabidopsis FLC* gene (Yan et al., 2004; Cooper et al., 2006; Dubcovsky et al., 2006). Both genes are most active at the onset of vernalization, exerting a strong repressive effect on flowering, which decreases parallel with the saturation of the vernalization requirement (Sheldon et al., 2000; Yan et al., 2004; Amasino, 2005; Dubcovsky et al., 2006). In the case of *FLC* this repressing effect was found to be linearly correlated with the transcript level. In barley, *VRN-H2* shows a similar relationship with the transcript level; when vernalization saturation is followed by a long photoperiod the *VRN-H2* gene is not completely repressed, but there is a quantitative delay in flowering (Dubcovsky et al., 2006; Karsai et al., 2006). Studies on gene activity, functional QTL analysis and segregating populations confirmed that the repression of flowering and the quantitative delaying effect of *VRN-H2* on flowering were primarily connected with its role in regulating *VRN-H1* gene activity (Dubcovsky et al., 2006; Kóti et al., 2006; Szűcs et al., 2007). Trevaskis et al. (2006) found that the activity of *VRN-H2* showed a diurnal rhythm, indicating that this gene is also subject to circadian regulation. The present work indicated that *VRN-H2* may also participate in the regulation of light intensity perception, a function that is independent of the *VRN-H1* gene. Based on recent results obtained in *Arabidopsis*, the *FLC* gene is involved not only in regulation pathways driven by vernalization, but also in the regulation of the circadian rhythm, partly by influencing the activity of photoreceptors that sense light quality and quantity, and partly by taking part in the temperature compensation of the circadian rhythm (McClung, 2006; Edwards et al., 2006; Salathia et al., 2006; Hotta et al., 2007; Zhou et al., 2007). The findings that both photoperiod and light intensity influence the role of the *VRN-H2* gene represent a strong parallelism with the *Arabidopsis FLC* gene, and indicate that the barley *VRN-H2* gene may also be involved in the regulation of the circadian rhythm.

The intron 1 region of the *VRN-H1* gene, which is critical for vernalization, is completely identical in the two parental lines of the barley mapping population (von Zitzewitz et al., 2005); both varieties (the facultative Dicktoo and the winter Kompolti Korai) carry the functional recessive allele, which can be repressed by the transcript of *VRN-H2* (Karsai et al., 2005). The flowering time QTL effects identified at the *VRN-H1* locus in field and controlled chamber tests demonstrate that the *VRN-H1* gene is also subject to regulation by environmental stimuli other than low temperature vernalization, and that the site(s) of this additional regulation is different from that of the vernalization regulation site (Karsai et al., 2005; Kóti et al., 2006; Szűcs et al., 2007). The recessive *VRN-H1* allele was found to be repressed by short photoperiod (Danyluk et al., 2003; von Zitzewitz et al., 2005). The novel information provided by the present work is that low light intensity differentially influences the activating effect of the two parental recessive alleles on flowering under an intermediate photoperiod regime.

In summary, low light intensity in association with photoperiod exerted a significant effect on the activity of the *VRN-H2* and *VRN-H1* vernalization response genes and on the relationship between them in barley. The modifying effect of low light intensity on gene functions was the strongest at photoperiods intermediate between short and long daylengths. The reactions of the two genes to low light intensity may imply that in addition to their role in vernalization-driven regulation, the *VRN-H2* gene may participate in the regulation of the circadian rhythm and the *VRN-H1* gene may be subject to circadian-driven developmental regulation.

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GENETIC VARIATION FOR STOMATAL CONDUCTANCE IN UPLAND COTTON AS INFLUENCED BY HEAT-STRESSED AND NON-STRESSED GROWING REGIMES

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Stomatal conductance is an important heat avoidance mechanism and its association with higher yield and heat resistance has been established in Pima cotton. Experiments were carried out on upland cotton under heat-stressed and non-stressed greenhouse and field regimes, to understand the impact of heat-stressed and non-stressed environments on the genetic and combining ability variations for stomatal conductance. The experimental material comprised 8 upland cotton cultivars and their 15 F_1 cross combinations obtained in a line × tester mating arrangement. The results showed high genetic variability for stomatal conductance in a single environment, but low genetic variability across environments, due to the higher magnitude of the environmental interaction, especially that caused by temperature regimes. The interaction effect of temperature regimes also substantially modified general and specific combining ability variations for stomatal conductance. The relative contributions of general and specific combining abilities to total phenotypic variation for stomatal conductance also underwent a great change across field temperature regimes. The non-stressed regime favoured the expression of genes causing the additive type of genetic variability. The heat-stressed field regime, however, favoured the expression of both additive and non-additive types of genetic variation for stomatal conductance in upland cotton. Recurrent selection for the accumulation of favourable genes for general combining ability under non-stressed conditions was suggested for improving stomatal conductance in applied cotton breeding programmes.

Key words: genetic variability, heat avoidance, stomatal conductance, temperature regimes

List of abbreviations: C (crosses), GCA (general combining ability), L (lines), P (parents), PAR (photosynthetically active radiation), R (temperature regimes), SCA (specific combining ability), T (testers), Y (years)

Introduction

Stomatal conductance has been identified as an important heat avoidance mechanism in crop plants. In Pima cotton, stomatal conductance is reported to be associated with higher yield and heat resistance (Radin et al., 1994; Lu et al., 1994; 1997). Stomatal conductance in Pima cotton is quantitative in nature (Percy et al., 1996). Genetically determined changes in stomatal conductance were observed as a result of the correlated response to selection for high yield and heat resistance in Pima cotton (Lu and Zeiger, 1994). In a progeny from a cross between *G. hirsutum* and *G. barbadense* cottons, the segregation pattern of stomatal conductance in the F_1 and F_2 populations also showed a clear genetic component (Lu et al., 1997), suggesting that stomatal conductance in cotton is amenable to genetic improvement. The feasibility of the direct genetic improvement of stomatal conductance is, however, not well understood. One of the shortcomings associated with such analysis is the well-documented environmental sensitivity of stomatal conductance. Jarvis and Mansfield (1981) and Zeiger et al. (1987) have given a good account of the environmental variables affecting stomatal conductance in crop plants. The present study was, therefore, initiated to understand the impact of heat-stressed and non-stressed regimes on genetic variability and the pattern of combining ability variation associated with the expression of stomatal conductance in upland cotton. Such information would be helpful in exploring the feasibility of direct genetic improvement in the mechanism of heat avoidance through enhanced stomatal conductance. The availability of an experimental system exhibiting genetic variation in stomatal conductance may also be helpful in elucidating the genetic regulation of stomatal conductance.

Materials and methods

The experimental material comprised 8 upland cotton cultivars (*Gossypium hirsutum* L.) varying in heat tolerance (Rahman et al., 2004), and their 15 F_1 cross combinations obtained in a line × tester pattern. Five cultivars were used as females (lines) and 3 as males (testers). The evaluation of the experimental material was carried out under heat-stressed and non-stressed greenhouse and natural field conditions at the Cotton Research Institute, Faisalabad, Pakistan.

Greenhouse experiment

A greenhouse experiment was conducted under two temperature regimes maintained in two separate chambers designated as non-stressed and heat-stressed regimes. The ideal temperature for cotton growth has been reported to be between 20 and 30°C (Reddy et al., 1998), while the ideal temperature for metabolic activity and photosynthesis was given as 23–33°C (Burke et al., 1988). A temperature regime of 35/21°C was selected to compensate for the relatively slower growth and reproduction under lower temperatures and to complete the experiments in non-stressed and heat-stressed chambers within the same number of days.

The heat-stressed chamber was maintained at a day/night temperature of 46/30°C ± 2°C and the non-stressed chamber at 35/21°C ± 2°C. The day length (light period) for both the chambers was 14 hours. The plants were grown in earthen pots (35 cm high, 30 cm diameter), each holding 9 kg soil (mixture of silt and peat in 3:1 ratio). Soil analysis was carried out before filling the pots. Soil properties were: EC, 0.59 dSm^{-1}; pH, 8.1; organic matter, 1.30%; saturation percentage, 29; available phosphorus, 30.1 ppm and potassium, 130 ppm. During the experiment, urea (46% nitrogen) was applied to the pots in solution form (10 g urea/litre of water) as irrigation water 30, 60 and 90 days after sowing.

The seeds were soaked in tap water for eight hours prior to sowing. Four seeds were sown in each pot at a depth of 2 cm. Later, two plants were retained in each pot and the remaining thinned out at the 2-true leaf stage. Each entry was represented by 6 pots (12 plants, 4 per replication) in each of the two chambers. The entries were arranged in a completely randomised fashion. The plants were allowed to grow under non-stressed temperatures (35/21°C ± 2°C) for 30 days after sowing. Later, the temperature in the heat-stressed chamber was gradually increased at an average rate of 2°C per day. Sunlight was the source of illumination in both the chambers, but during the morning and evening hours, fluorescent bulbs were used to supplement the light period and intensity. PAR (photosynthetically active radiation) in both the chambers ranged between 1400 and 1600 μmol m^{-2} s^{-1} at noon. The relative humidity varied between 65 and 80% throughout the experimental period in both the chambers. The pots were watered with 400 ml water on alternate days before and after peak flowering and daily during the peak flowering period. Peak flowering was the period during which the cotton plants produced maximum flowers (between 50 to 70 days after sowing in the greenhouse, and 70 to 90 days in the field experiments). Care was taken to avoid drought or over-saturation.

Field experiments

Field experiments were carried out during the 2000 and 2001 crop seasons. The experiments were sown on two dates, 7th April and 29th May 2000, 15th April and 4th June 2001, to provide two temperature regimes, especially during the reproductive stage. April sowing was selected to synchronise the peak flowering period with the highest temperatures of the year in June–July. The minimum and maximum temperatures received by the crop in the early-sown (April) experiments were significantly higher than those received by the crop in the late-sown (June) experiments (Rahman et al., 2004) and were therefore taken as heat-stressed and non-stressed field regimes, respectively. The layout design for all the field experiments was a randomised complete block with three replications. The plot size in each replication measured 450 × 75 cm, and accommodated 16 plants spaced 30 cm apart. Both experiments were sprayed for proper insect control when required.

Determination of stomatal conductance

Stomatal conductance in the greenhouse and field experiments was measured with a portable steady-state Porometer (PMR-2, PP Systems, UK) during the maximum flowering and fruiting periods of crop growth.

Environmental conditions during data recording

In the greenhouse, measurements were recorded between 1200 and 1400 hours and in the field between 1300 and 1500 hours. At the time of data recording, the photosynthetically active radiation (PAR) varied between 1300–1400 and 1600–1700 μmol $m^{-2}s^{-1}$, and the relative humidity from 60–65% and 45–50% in the greenhouse and field experiments, respectively. The ambient CO_2 concentration ranged from 340 to 351 μmol mol^{-1} in the field and 320 to 324 μmol mol^{-1} in the greenhouse. During data recording, the air temperature in the field ranged between 42 and 44°C in both the years. The temperature of the Porometer was 28.3–35.8°C during data recording in all the experiments. The inlet flow rate was adjusted to 45–50 cm^3/min, which was kept adjusted to give at least a 10% relative humidity difference between inlet and outlet.

The time of data recording (1100–1300 hours in the greenhouse, 1200–1400 hours in the field on clear sunny days) was chosen because at this time the plants suffer maximum heat stress, making maximum phenotypic differences between the genotypes apparent (Lu et al., 1994). To minimise the confounding effect of drought on stomatal conductance, care was taken to provide adequate water to the experimental materials. In the greenhouse, pots were watered daily in the afternoon, and in the field experiments stomatal conductance was recorded on the 4th, 5th and 6th day after irrigation to provide field capacity at the time of data recording. Stomatal conductance was recorded on the youngest fully expanded (20 to 23-day-old) leaves, growing at the 5th or 6th node on the main stem. The leaves were tagged on the day they unfolded, which was counted as day 1. Leaves were tagged on different days, so that leaves of the required age were always available for the cyclic measurement of the data. Due to the small time window of maximum heat stress during the day, at which maximum phenotypic differences could be observed, the data were recorded in a cyclical manner and sample sizes were kept limited. Data were recorded from a maximum of two plants from each generation in each cycle. After completing the required number of plants from each genotype and generation, the next cycle was started. The sample size for each entry was 3 plants per replication in the greenhouse and 5 plants per replication in the field experiments.

Statistical and biometrical procedures

The data were analysed in a factorial arrangement. The homogeneity of the variances was tested before making combined analyses of variance across years and temperature regimes. Parents and crosses were treated separately. Variation between the crosses was partitioned into that due to lines, testers and lines × testers. Likewise, variation due to cross × year and cross × temperature regime interactions were partitioned into their subcomponents. Variation due to lines and testers was interpreted as general combining ability (GCA) variation and that due to lines × testers as specific combining ability (SCA). The effect of years and temperature regimes was assumed to be fixed, and that of genotypes (parents and crosses) random, since the aim was to draw conclusions on the cotton germplasm from which the parental cultivars were sampled. The combining ability effect associated with each parent was determined following Kempthorne (1957), as illustrated by Singh and Chaudhary (1977). Genetic variability across environments was determined as:

Genetic variance for greenhouse experiment = $\sigma^2_G = MS5 - MS3 / r * R$

Genetic variance for field experiments = $\sigma^2_G = MS5 - MS4 - MS3 + MS5 / r * Y * R$ (Table 1), where MS values were the mean squares depicted in Table 1 and r, Y and R were the number of replications, years and temperature regimes, respectively.

Results

Variation between parental cultivars for stomatal conductance

The temperature regimes were a significant source of variation for stomatal conductance between the parental cultivars both in the greenhouse and field (Table 1). The rankings of the parental cultivars for stomatal conductance also shifted across temperature regimes. As a result of the significant parent × temperature regime interaction, differences between parental cultivars were not evident across temperature regimes in the greenhouse. The magnitude of phenotypic variation between parental cultivars in the greenhouse experiment was almost twice as high as in the field experiment, but the genetic variability was zero (negative) due to the excessively higher magnitude of the C × R interaction variance (Table 1).

Combining ability variation for stomatal conductance

Analysis of variance indicated the significant effect of the temperature regimes ($P<0.01$) on the expression of stomatal conductance between the crosses under both greenhouse and field conditions (Table 2). The crosses × temperature regime interaction was also significant in both the greenhouse and field experiments ($P<0.05$), indicating that in both environments the temperature regime significantly modified the relative expression of stomatal conductance between the crosses. The great magnitude of the interactions, particularly the C × R interaction, caused genetic variability to be low under both greenhouse and field conditions.

Table 1

Analysis of variance for stomatal conductance (mol $m^{-2} s^{-1}$) between parental cultivars, across temperature regimes in the greenhouse, and across years and temperature regimes in the field

Greenhouse			Field			
Source	df	Mean squares	Source	df		Mean squares
Temp. regimes (R)	1	0.187**	Replication	2		0.085**
Parents (P)	7	0.012^{ns}	Years (Y)	1		0.138**
P × R	7	0.013*	Temp. regimes (R)	1		9.779**
Residuals	32	0.006	Y × R	1		0.001^{ns}
Total	47		Parents (P)	7	MS5	0.065*
CV%		15.342	P × Y	7	MS4	0.021^{ns}
σ^2_G		0.000	P × R	7	MS3	0.079*
			P × Y × R	7	MS2	0.017*
			Residuals	62	MS1	0.008
			Total	95		
			σ^2_G			0.000

*, ** significant at the 5 and 1% levels of probability, respectively; ns = non-significant (P>0.05)

Table 2

Analysis of variance in combining ability for stomatal conductance (mol $m^{-2} s^{-1}$) across temperature regimes in the greenhouse and across years and temperature regimes in the field

Greenhouse			Field		
Source	df	Mean squares	Source	df	Mean squares
Temp. regimes (R)	1	0.480**	Replication	2	0.009^{ns}
Crosses (C)	14	0.017**	Years (Y)	1	0.437**
Lines (L)	4	0.032^{ns}	Temp. regimes (R)	1	19.468**
Testers (T)	2	0.004^{ns}	Y × R	1	0.050**
Lines × Testers	8	0.013**	Crosses (C)	14	0.076 *
C × R	14	0.020**	Lines (L)	4	0.125**
L × R	4	0.029**	Testers (T)	2	0.149**
T × R	2	0.012**	Lines × Testers	8	0.032**
L × T × R	8	0.017**	C × Y	14	0.093^{ns}
Residuals	60	0.005	L × Y	4	0.037^{ns}
Total	89		T × Y	2	0.027^{ns}
			L × T × Y	8	0.013^{ns}
			C × R	14	0.090**
			L × R	4	0.087**
			T × R	2	0.221**
			L × T × R	8	0.059**
			C × Y × R	14	0.083**
			L × Y × R	4	0.105**
			T × Y × R	2	0.169**
			L × T × Y × R	8	0.051**
			Residuals		0.014

*, ** significant at the 5 and 1% levels of probability, respectively; ns = non-significant (P>0.05)

The interaction of the temperature regimes with lines, tester, and lines × testers was also significant ($P<0.01$), indicating that the temperature regime substantially modified the general and specific combining ability variations for stomatal conductance both in the greenhouse and in the field. Because the C × R interaction was significant in both the greenhouse and field experiments, analyses of combining ability were also run separately for heat-stressed and non-stressed regimes (Table 3). The results revealed significant differences between the crosses for stomatal conductance under heat-stressed and non-stressed greenhouse and field regimes. Variation in stomatal conductance due to lines × testers (specific combining ability) was significant for the heat-stressed field regime and for both the heat-stressed and non-stressed greenhouse regimes. Variation due to lines was significant ($P<0.01$) in the non-stressed field regime and that due to testers in the case of heat stress. On quantitative grounds, however, the relative contribution of specific combining ability (variation due to lines × testers) accounted for 47% of the total variation in stomatal conductance in both the heat-stressed and non-stressed greenhouse regimes. The relative contribution of general combining ability from lines accounted for 52% in the heat-stressed greenhouse regime and 44% in the non-stressed regime. The relative contributions of general and specific combining abilities to the total phenotypic variation for stomatal conductance, however, underwent a great change across the field temperature regimes (Table 4). The relative contribution of specific combining ability and general combining ability from testers decreased from 41 and 47%, respectively, in the heat-stressed field regime to only 11% in each case in the non-stressed regime. By contrast, the relative contribution of general combining ability from lines increased from 13% under heat-stressed conditions to 80% in the non-stressed regime. This suggested that the non-stressed field regime was conducive for the expression of genes controlling general combining ability in general and the general combining ability of lines in particular. It could, therefore, be inferred that the expression of stomatal conductance in upland cotton was predominantly controlled by the additive type of genetic variability under non-stressed field conditions and by both additive and non-additive types of genetic variation under heat-stressed field conditions.

Status of parental cultivars for general combining ability (GCA) effects

General combining ability effects in the greenhouse regimes (Table 5) revealed relatively low variation for stomatal conductance between the parental cultivars in the presence and absence of heat stress. Cultivar FH-634, despite having below-average stomatal conductance, was the only parent that exhibited a significantly positive GCA effect for stomatal conductance in both heat-stressed and non-stressed regimes. This indicated that cultivar FH-634 possessed genes or a gene complex controlling high stomatal conductance, but that these genes were not expressed in the parental generation, though they were transferred to its progenies.

Table 3

Analysis of variance in combining ability for stomatal conductance (mol m^{-2} s^{-1}) in heat-stressed and non-stressed regimes in the greenhouse and field

Source	df	Mean squares (greenhouse)		df	Mean squares (field)	
		Heat-stressed	Non-stressed		Heat-stressed	Non-stressed
Replications				2	0.01^{ns}	0.02^{ns}
Crosses	14	0.018**	0.018**	14	0.11**	0.05 *
Lines (L)	4	0.033^{ns}	0.028^{ns}	4	0.05^{ns}	0.14**
Testers (T)	2	0.002^{ns}	0.014^{ns}	2	0.36 *	0.01^{ns}
L × T	8	0.015 *	0.015**	8	0.08**	0.01^{ns}
Residuals	30	0.007	0.003	28	0.02	0.02
Total	44			44		

*, ** significant at the 5 and 1% levels of probability, respectively; ns = non-significant (P>0.05)

Table 4

Estimates of specific combining ability effects associated with upland cotton crosses obtained in the line × tester model for stomatal conductance (mol m^{-2} s^{-1}), following line × tester analysis of combining ability in April (heat-stressed) and June (non-stressed) regimes in the field

F_1 cross	Greenhouse		Field	
	Heat-stressed	Non-stressed	Heat-stressed	Non-stressed
C16	-0.009^{ns}	0.051^{ns}	0.207**	0.020^{ns}
C17	-0.024^{ns}	-0.030^{ns}	0.024^{ns}	-0.075^{ns}
C18	0.033^{ns}	-0.021^{ns}	−0.231**	0.055^{ns}
C26	0.099 *	0.009^{ns}	-0.025^{ns}	0.023^{ns}
C27	-0.020^{ns}	-0.048^{ns}	−0.156 *	0.076^{ns}
C28	-0.079^{ns}	0.039^{ns}	0.180**	-0.099^{ns}
C36	0.029^{ns}	−0.065 *	-0.040^{ns}	0.056^{ns}
C37	-0.043^{ns}	-0.014^{ns}	−0.157 *	0.044^{ns}
C38	0.014^{ns}	0.079 *	0.197**	-0.100^{ns}
C46	-0.109^{ns}	0.035^{ns}	-0.071^{ns}	0.032^{ns}
C47	0.060^{ns}	0.075 *	0.322**	-0.050^{ns}
C48	0.050^{ns}	-0.110^{ns}	−0.251**	0.018^{ns}
C56	-0.010^{ns}	-0.031^{ns}	-0.071^{ns}	−0.132 *
C57	0.027^{ns}	0.017^{ns}	-0.033^{ns}	0.005^{ns}
C58	-0.017^{ns}	0.014^{ns}	0.105^{ns}	0.127^{ns}
SE for SCA	0.047	0.033	0.068	0.067

*, ** significant at the 5 and 1% levels of probability, respectively; ns = non-significant (P>0.05)

Three parental cultivars, MNH-552, FH-900 and CIM-443, appeared to be more sensitive to temperature regimes in terms of GCA effects. Cultivar FH-900 had high stomatal conductance but a poor GCA effect under non-stressed greenhouse conditions and a good GCA effect in the non-stressed field regime. Cultivar CIM-443, with low mean stomatal conductance, exhibited poor GCA effects under heat-stressed greenhouse and non-stressed field conditions and a good GCA effect in the heat-stressed field regime. Similarly, cultivar MNH-552, with high stomatal conductance, had a good GCA effect under non-stressed greenhouse conditions and a poor GCA effect when heat-stressed in the field.

Since the experiments were not aimed at finding specific combinations for stomatal conductance, the specific combining ability effects associated with each cross combination were not calculated.

Table 5

Estimates of general combining ability effects associated with upland cotton cultivars for stomatal conductance (mol m^{-2} s^{-1}) following line × tester analysis of combining ability in various temperature regimes in the greenhouse and field

Cultivars	Greenhouse				Field			
	Heat-stressed		Non-stressed		Heat-stressed		Non-stressed	
	GCA	Mean	GCA	Mean	GCA	Mean	GCA	Mean
FH-634	0.09**	0.33	–0.03^{ns}	0.55	0.13**	0.75	0.24**	1.33
FH-900	–0.02^{ns}	0.39	–0.05 *	0.59	–0.04^{ns}	0.83	0.10 *	1.55
MNH-552	0.00^{ns}	0.49	0.09**	0.56	–0.17**	0.86	–0.01^{ns}	1.55
CIM-448	0.02^{ns}	0.52	0.02^{ns}	0.56	–0.03^{ns}	0.69	–0.15**	1.51
CIM-443	–0.08**	0.37	–0.02^{ns}	0.53	0.11**	0.82	–0.19**	1.52
Karishma	0.01^{ns}	0.40	–0.02^{ns}	0.46	–0.11**	0.87	0.05^{ns}	1.29
CRIS-19	–0.01^{ns}	0.54	0.03 *	0.55	0.25**	0.78	–0.03^{ns}	1.38
HR109-RT	0.01^{ns}	0.37	–0.02^{ns}	0.62	–0.14**	0.72	–0.02^{ns}	1.35
SE gca (L)	0.03		0.02		0.04		0.04	
SE gca (T)	0.02		0.01		0.03		0.03	
CD 5%		0.15		0.09		0.13		0.21

*, ** significant at the 5 and 1% levels of probability, respectively; ns = non-significant (P>0.05)

Discussion

The analysis of the historical series of Pima cotton cultivars has demonstrated that selection for high yield and heat resistance has also led to genetic improvement for stomatal conductance (Cornish et al., 1991; Lu and Zeiger, 1994; Lu et al., 1994; Radin et al., 1994). Results from another investigation on stomatal conductance in Pima cotton (Faver et al., 1997) indicated that breeding for yield has improved photosynthetic capacity and stomatal conductance in advanced cultivars under conditions of water stress. These researchers further opined that upland cotton could be used as a source of genetic variation for higher stomatal conductance in Pima breeding programmes. The present investigation has revealed that although a significant level of phenotypic variation for stomatal conductance was available in upland cotton cultivars, genetic variability was only available for individual environments, not across environments. This was due to the presence of higher interactions between genotypes and, particularly, the temperature regime. These results were in line with the well-documented sensitivity of stomatal conductance to environmental conditions (Jarvis and Mansfield, 1981; Zeiger et al., 1987). General and specific combining ability variations for stomatal conductance were also liable to modification across temperature regimes, requiring the determination of an appropriate temperature regime, where favourable combining ability variations could be obtained. From the breeding point of view, recurrent selection for general combining ability under non-stressed conditions might be helpful in accumulating favourable genes for higher stomatal conductance. Selection for higher stomatal conductance should of course be combined with selection for yield.

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SACCHARIDE AND FRUCTOOLIGOSACCHARIDE ACCUMULATION ACROSS LEAF-BASES DURING GROWTH AND BULB DEVELOPMENT OF ONION (*Allium cepa* L.)

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The accumulation of saccharides and fructooligosaccharides (FOS) in the individual leaf-bases of onion (*Allium cepa* L.) was investigated during growth and bulb development. Saccharides and FOS were analysed by means of high performance anion exchange chromatography-pulsed amperometric detection (HPAEC-PAD). The glucose content was the highest, while the content of saccharides (glucose, fructose and sucrose) increased during June, July and August and decreased slightly during September. The tri-saccharides all accumulated to a similar extent, although the neokestose [**3b**, 6^G-β-D fructofuranosylsucrose] content was higher than that of 1-kestose [**3a**, 1^F-β-D-fructofuranosylsucrose]. Tetra-, penta- and high-DP (degree of polymerization) FOS also showed a similar pattern, though the contents of **4b** [6^G (1-β-D-fructofuranosyl)$_2$ sucrose] and **5b** [6^G (1-β-D-fructofuranosyl)$_3$ sucrose] were higher compared with that of other tetra- [**4a**, 1^F (1-β-D-fructofuranosyl)$_2$ sucrose and **4c**, 1^F, 6^G-di-β-D-fructofuranosyl sucrose] and penta-saccharides [**5a**, 1^F (1-β-D-fructofuranosyl)$_3$ sucrose]. Total FOS accumulated to a greater extent in the inner (youngest) leaf-bases than in the outer (oldest) leaf-bases, and their content was high during August. The total carbohydrates content was 6.71, 7.25, 8.10 and 6.30 g 100 g^{-1} FW during June, July, August and September, respectively. During bulb formation, a balance was observed between the glucose, fructose, sucrose and FOS contents, with an average ratio of 20:10:10:60 of total carbohydrates, respectively.

Key words: saccharides, fructooligosaccharides, leaf-bases, accumulation, growth, *Allium cepa*

Introduction

About 80% of bulb dry matter consists of non-structural carbohydrates (Darbyshire and Henry, 1981). The most predominant of these non-structural carbohydrates is glucose, followed by fructose, sucrose and low-molecular-weight fructans, while starch and raffinose are absent (Darbyshire and Henry, 1981; Benkeblia et al., 2002).

The fructooligosaccharides (FOS), polyfructosylsucroses of varying molecular size, are the main carbohydrate reserve of onion. They accumulate during bulbing and are then catabolized during regrowth and the sprout development of the bulbs (Darbyshire, 1978). FOS may have functions other than carbon storage: they have been implicated in protecting plants against water deficit by drought or low temperature (Hendry, 1993; Hendry and Wallace, 1993; Vijn and Smeekens, 1999), or acting as osmoregulators (Hendry, 1993; Livingston and Henson, 1998; Hincha et al., 2000), and they have an important impact on the storability of onion bulbs (Rutherford and Whittle, 1984).

During the growth and bulbing of the onion plant, the leaf scales thicken and form the characteristics of the bulb. At the onset of bulbing, the leaf sheaths swell, bladeless bulb scales are initiated and these swell to form the central storage tissues and accumulation sites of FOS. Bulb formation and subsequent growth are influenced by photoperiod and temperature (Brewster, 1977), and the bulbing process is initiated mainly by long days (Lercari, 1982; Lancaster et al., 1996; Tei et al., 1996) and high temperatures (Lancaster et al., 1996; Kato, 1964). Clearly, many factors other than photoperiod and temperature affect bulbing, e.g. cultural conditions (Brewster, 1995) and climate change (Wurr et al., 1998).

The biochemical pathway of FOS synthesis in liliaceous plants was well described by Shiomi (1989) and Fujishima et al. (2005). Moreover, a substantial literature exists on the variation of FOS in onion bulbs during the post-harvest life of the bulbs. In spite of this abundance of investigations focused on this aspect, few or none described the content of different FOS during the bulbing of onion, especially across different scales of the bulb tissues. A recent investigation reported the carbohydrate chemistry of the onion leaf bases (Ng et al., 1998), and Darbyshire and Henry (1978) investigated the non-structural carbohydrate content of individual leaf-bases of onion and noted that total fructan concentration decreased from the youngest (inner) to the oldest (outer) leaf-bases of the bulb. These results are in agreement with the results of Jaime et al. (2001). However, values for the variation of FOS during bulbing, as well as their variation across leaf bases, are not readily available in the current literature. Thus, the objective of this study was to investigate variation in the accumulation of saccharides and different FOS in onion leaf-bases during the bulbing period.

Materials and methods

Plant materials and growth conditions

Onion bulbs (*Allium cepa* var. Sapporo H1, summer cultivar) were cultivated in the University Farm, Ebetsu, Hokkaido, Japan, where the average daylength in June, July, August and September is 15, 15, 14 and 13 h, the average temperature 12, 16, 20 and 18°C and the average rainfall 122, 160, 198 and 173 mm, respectively. These climatic data are the averages of the past five years (from 2000 to 2004). The seeds were sown on March 3 and the seedlings were transplanted on May 8. The bulbs were sampled on June 13 (3 leaf-bases), July 14 (6 leaf-bases) and August 13 (8 leaf-bases), with the final sampling at harvest on September 8. The leaves were numbered from 1 (outer, older ones) to 8 (inner, younger ones). After separation, leaf tissue samples were stored at –40°C until use.

Fructooligosaccharide extraction

Fructooligosaccharides (FOS) were extracted by the method of Shiomi (1992). Leaf tissue (10 g) was homogenized in 80 ml of aqueous ethanol (70%) using a small amount of calcium carbonate (0.5 g L^{-1}). The homogenate was boiled under reflux in a water bath for 10 min. Then the homogenate was filtered and the residue was extracted three times with aqueous ethanol and once with water under the same conditions. The filtrates were combined and made up to 500 ml with distilled water. An aliquot of the filtrate (10 ml) was concentrated to dryness under a vacuum at 35ºC using a Büchi rotavapor (Büch Laboratoriums-Technik, Flawil, Switzerland). The concentrated sugars were collected in one ml of water, passed through a 0.45 μm filter and analysed by high performance anion exchange chromatography (HPAEC, Dionex, Sunnyvale, CA, USA). All processes were run in triplicate.

Fructooligosaccharide analysis

FOS were separated on an HPLC-carbohydrate column PA1, Carbo Pack with a Dionex Bio LC series HPLC (Sunnyvale, CA, USA) and pulsed amperometric detector (PAD). The gradient was established by mixing eluent A (150 mM NaOH) with eluent B (500 mM Na acetate in 150 mM NaOH) in two ways. System I: 0–1 min, 25 mM; 1–2 min, 25–50 mM; 2–20 min, 50–200 mM; 20–22 min, 500 mM; 22–30 min, 25 mM. System II: 0–1 min, 25 mM; 1–2 min, 25–50 mM; 2–14 min, 50–500 mM; 14–22 min, 500 mM; 22–30 min, 25 mM. The flow rate through the column was 1.0 ml min^{-1}. The applied PAD potentials for E1 (500 ms), E2 (100 ms) and E3 (50 ms) were 0.01, 0.60 and –0.60 V, respectively, and the output range was 1 μC. Fructooligosaccharides were expressed in g per 100 g fresh weight (g 100 g^{-1} FW). System I was used for the separation of oligosaccharides, and system II for that of oligo- and polysaccharides.

Glucose, fructose and sucrose standards were purchased from Nacalai Tesque Inc. (Kyoto, Japan). 1-Kestose [**3a**, 1^F-β-D-fructofuranosylsucrose, 1-kestotriose] and nystose [**4a**, 1^F (1-β-D-fructofuranosyl)$_2$ sucrose, 1,1-kestotetraose] were previously prepared in the laboratory as described by Takeda et al. (1994). Neokestose [**3b**, 6^G-β-D-fructofuranosylsucrose, 6G-kestotriose], **4b** [6^G (1-β-D-fructofuranosyl)$_2$ sucrose, 1, 6G-kestotetraose], **4c** [1^F, 6^G–di-β-D-fructofuranosyl sucrose, 1 and 6G-kestotetraose], **5a** [1^F (1-β-D-fructofuranosyl)$_3$ sucrose, 1, 1, 1-kestopentaose), **5b** [6^G (1-β-D-fructofuranosyl)$_3$ sucrose, 1,1,6G-kestopentaose], **5c** [1^F (1-β-D-fructofuranosyl)$_2$-6^G-β-D-fructofuranosyl sucrose, 1,1 and 6G-kestopentaose], **5d** [1^F-β-D-fructofuranosyl-6^G (1-β-D-fructofuranosyl)$_2$ sucrose, 1 and 1,6G-kestopentaose], DP 6 saccharides, [1^F (1-β-D-fructofuranosyl)$_m$- 6^G (1-β-D-fructofuranosyl)$_n$ sucrose; **6b**: $m = 0$, $n = 4$ (1,1,1,6G-kestohexaose); **6c**: $m = 3$, $n = 1$ (1,1,1 and 6G-kestohexaose); **6d$_1$**: $m = 1$, $n = 3$ (1 and 1,1,6G-kestohexaose); **6d$_2$**: $m = 2$, $n = 2$ (1,1 and 1,6G-kestohexaose)] and DP (degree of polymerisation) up to 12 were obtained from asparagus roots as described in previous papers (Shiomi et al., 1976; 1979; Shiomi, 1981). The standards **6a** [1^F (1-β-D-fructofuranosyl)$_4$ sucrose, 1,1,1,1-kestohexaose) and **7a** [1^F (1-β-D-fructofuranosyl)$_5$ sucrose, 1,1,1,1,1-kestoheptaose)] were prepared from Jerusalem artichoke tubers in our laboratory. Because the nomenclature of fructans is not simple due to the very complex structures, the nomenclatures for FOS proposed by Lewis (1993) and Waterhouse and Chatterton (1993) were also used. All isolated and synthesized standards were of high grade purity ($\geq$ 99.8%).

Statistical analysis

All determinations were carried out in triplicate (three bulbs were sampled per assay date) and expressed on a fresh weight (FW) basis. The experiment was repeated twice and the data averaged. Data analyses were performed using Graph Pad InStat 3.06 (GraphPad Software, Inc. San Diego, CA, USA) and LSD (at $P < 0.05$) was calculated. Total carbohydrate means were compared by Student's *t*-test (at $P < 0.05$) using the same software.

Results

The content of mono- and disaccharides is shown in Figure 1. At the three leaf-bases stage, glucose ranged from 1.20 to 1.50 g 100 g^{-1} FW. Afterwards, glucose increased during July and August and ranged from 1.40 to 2.20 and from 1.30 to 1.70 g 100 g^{-1} FW, respectively. During September, the glucose content across the leaf-bases decreased slightly and ranged from 0.80 to 1.50 g. The fructose content was low during June (0.30 to 0.70 g) and increased four to six times (1.10 to 1.80 g) during July, then decreased and remained stable during August and September, ranging from 0.40 to 1.00 g 100 g^{-1} FW.

Surprisingly, the sucrose content varied slightly during bulb formation and did not accumulate in the leaf-bases. The sucrose content in June, July, August and September varied from 0.60 to 0.85 g, 0.55 to 1.20 g, 0.70 to 1.10 g, and 0.50 to 0.75 g 100 g^{-1} FW, respectively.

The content of tri-FOS isomers showed a different pattern, as illustrated in Figure 2. 1-Kestose (**3a**) was higher during June and ranged from 0.40 to 0.60 g 100 g^{-1} FW, while during July, August and September it showed a steady state, ranging from 0.20 to 0.35 g 100 g^{-1} FW. The neokestose (**3b**) content was high during June, ranging from 0.70 to 0.90 g, then decreased during July. In August, the content increased to the levels observed during June. Afterwards, neokestose decreased in September to values of 0.20 to 0.30 g 100 g^{-1} FW. Total trisaccharides were higher during June and August, but were lower during July and September. It was also noted that the inner leaf-bases (youngest ones) showed high levels of trisaccharides, especially during August.

The nystose (**4a**) content was highest in June, decreasing to, 0.10 to 0.15 g in July, 0.15 to 0.23 g in August, and 0.10 to 0.15 g 100 g^{-1} FW in September (Fig. 3). The tetra-isomer **4b** content was higher, varying from 0.50 to 0.60 g during June, while it decreased to 0.12 to 0.30 g 100 g^{-1} FW during July. During August, **4b** increased again, ranging from 0.23 to 0.55 g 100 g^{-1} FW, while decreasing during September to 0.10 to 0.25 g 100 g^{-1} FW. Isomer **4c** showed a similar pattern to **4b,** with values of 0.40 to 0.55 g, 0.10 to 0.21 g, 0.23 to 0.55 g, and 0.10 to 0.17 g 100 g^{-1} FW during June, July, August and September, respectively. The total tetra-saccharides showed a similar pattern, and their content was high during June and August, ranging from 1.20 to 1.45 g and 0.57 to 1.13 g 100 g^{-1} FW, respectively, while decreasing to about half during July and September, with values of 0.28 to 0.53 g and 0.34 to 0.55 g 100 g^{-1} FW, respectively. Nevertheless, a similar content was noted in the inner leaf-bases, especially for isomers **4b** and **4c**, during August and September.

The content of penta-saccharides is illustrated in Figure 4. During June, isomers **5a** and **5b** ranged from 0.12 to 0.16 g and 0.32 to 0.40 g 100 g^{-1} FW, respectively. Afterwards, **5a** decreased during July and August to 0.05 to 0.15 g 100 g^{-1} FW, while **5b** decreased during July but increased during August, ranging from 0.12 to 0.27 g 100 g^{-1} FW. Isomers **5c** + **5d** showed a similar pattern, ranging from 0.68 to 0.75 g, 0.10 to 0.18 g, 0.15 to 0.53 g and 0.10 to 0.14 g 100 g^{-1} FW during June, July, August and September, respectively. Thus, the total penta-saccharides varied in the same manner, with values of 1.14 to 1.31 g, 0.25 to 0.45 g, 0.34 to 0.90 g and 0.30 to 0.54 g 100 g^{-1} FW during June, July, August and September, respectively. The youngest (inner) leaf-bases also showed a higher amount of penta-saccharides than the oldest (outer) ones.

High-DP FOS (6 and higher) varied similarly and a difference between the inner and outer scales was also observed, as illustrated in Figure 5. Values varied from 0.20 to 0.30 g, 0.15 to 0.40 g, 0.20 to 0.35 g and 0.20 to 0.24 g 100 g^{-1} FW during June, July, August and September, respectively.

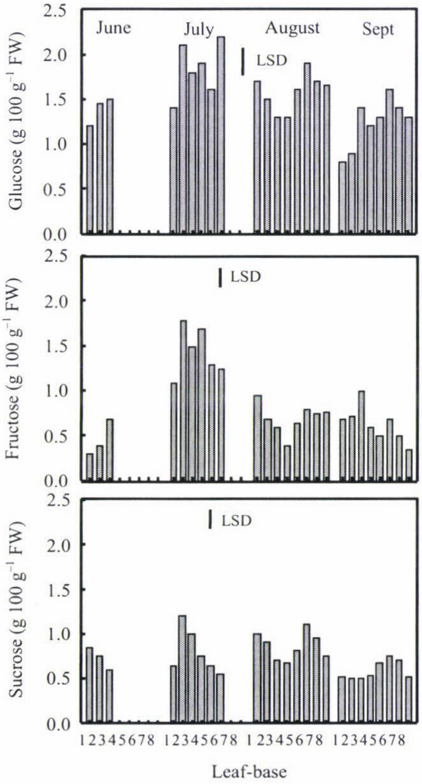

Fig. 1. Accumulation of glucose, fructose and sucrose across leaf-bases during bulbing of onion (LSD at $P < 0.05$) (Numbers 1 → 8 refer to inner (youngest) → outer (oldest) leaf-bases of the bulb)

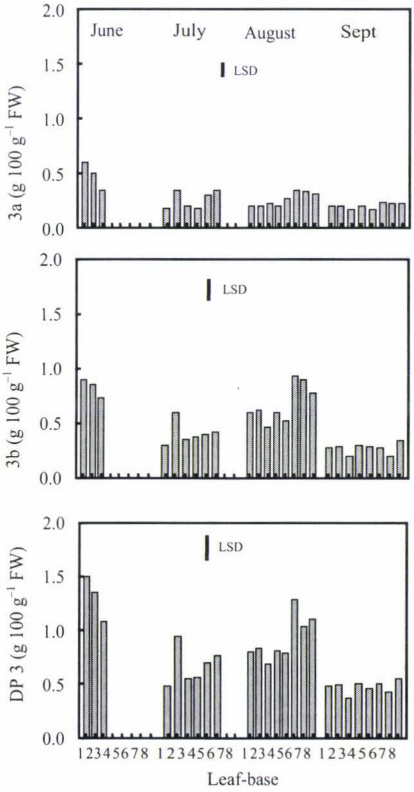

Fig. 2. Accumulation of trisaccharides across leaf-bases during bulbing of onion (LSD at $P < 0.05$) (Numbers 1 → 8 refer to inner (youngest) → outer (oldest) leaf-bases of the bulb)

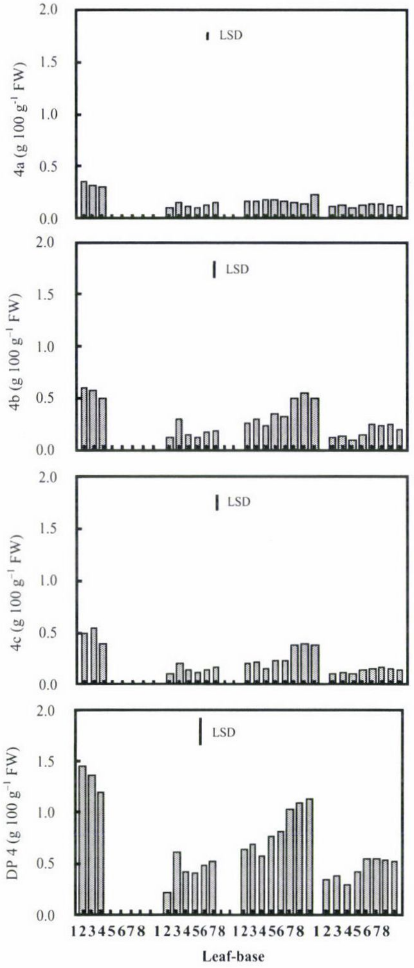

Fig. 3. Accumulation of tetra-saccharides across leaf-bases during bulbing of onion (LSD at $P < 0.05$) (Numbers 1 → 8 refer to inner (youngest) → outer (oldest) leaf-bases of the bulb)

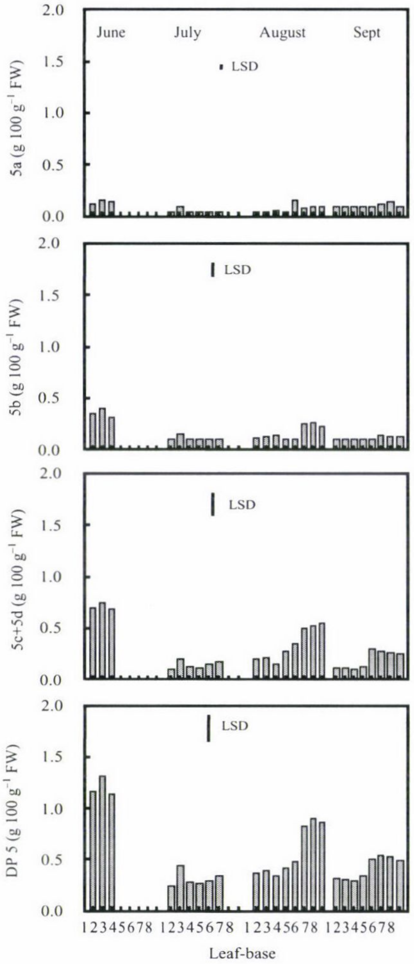

Fig. 4. Accumulation of penta-saccharides across leaf-bases during bulbing of onion (LSD at $P < 0.05$) (Numbers 1 → 8 refer to inner (youngest) → outer (oldest) leaf-bases of the bulb)

The total carbohydrate content showed a pattern close to that of high-DP FOS with a high level in June, ranging from 6.70 to 7.80 g 100 g^{-1} FW (Fig. 6), decreasing during July to 3.60 to 6.00 g, then increasing again to between 4.00 (outer leaf-bases) and 7.80 g (inner leaf-bases) during August. Afterwards, the total carbohydrates content decreased during September and ranged from 2.40 to 5.30 g 100 g^{-1} FW. The average carbohydrate content was 6.71, 7.25, 8.10 and 6.30 g 100 g^{-1} FW during June, July, August and September, respectively. Surprisingly, a balance between mono- and di-saccharides and high-DP FOS was observed during FOS accumulation, except for July, when the FOS content was slightly lower compared to that in June, August and September. These balanced contents were approximately 20%, 10%, 10% and 60% for glucose, fructose, sucrose and FOS, respectively. On the other hand, mono-, di- and fructooligosaccharides were seen to decrease during September.

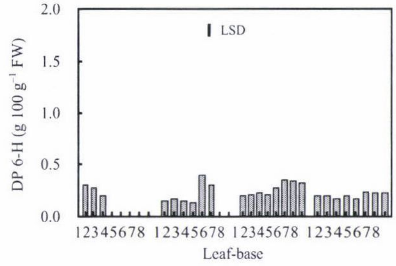

Fig. 5. Accumulation of DP 6 and higher (H) fructooligosaccharides across leaf-bases during bulbing of onion (LSD at $P < 0.05$) (Numbers 1 → 8 refer to inner (youngest) → outer (oldest) leaf-bases of the bulb)

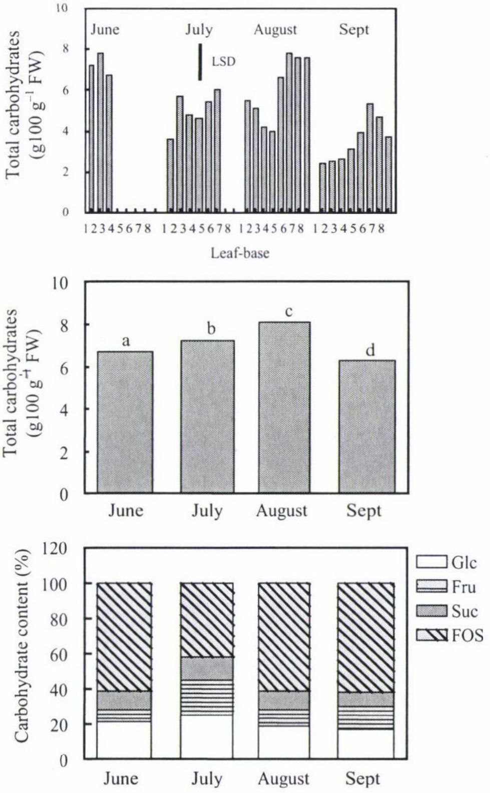

Fig. 6. Total accumulated carbohydrates across leaf-bases, and total carbohydrate content accumulated during bulbing of onion (in second figure, bars with different letters are significantly different at $P < 0.05$) (Top figure: Numbers 1 → 8 refer to inner (youngest) → outer (oldest) leaf-bases of the bulb)

Discussion

Up to now, few studies have described the distribution of carbohydrates and FOS in onion bulb tissues, while none has considered the accumulation and distribution of FOS across leaf-bases and during bulb formation, except for a study by Shiomi et al. (1997).

First, Bacon (1959) examined the distribution of smaller fructans (up to DP 5) in mature onions and reported that tetra- and penta-saccharides are absent from the outer leaf-bases and increase to maximum concentration in the inner leaf-bases. Later de Miniac (1970) reported a similar distribution of the tri-, tetra- and penta-saccharides, except that DP 3 and DP 4 were present in the outer leaf-bases. Darbyshire and Henry (1978) investigated the non-structural carbohydrates content of individual leaf-bases of mature harvested onion bulbs and noted that DP 3 to 9 fructans were present in all leaf-bases and increased in concentration towards the younger leaf-bases. Thus, with the increasing DP, the concentration of each fructan declined and this relationship was consistent for all leaf-bases. These authors also noted that the glucose and sucrose levels tended to remain constant, while the fructose level was high in the inner leaf-bases. All these results are in agreement with those reported here, except for fructose, which tended to vary across the leaf-bases, as shown in Figure 3.

Recently, Ng et al. (1998) performed a comparative examination of the cell wall chemistry of different component tissues of five varieties of onion bulbs. They dissected the onion bulb into four different tissue regions [top+bottom, brown dry outer skins, outer fleshy leaves and inner fleshy leaves, respectively] and noted that the inner leaf-bases contained more soluble polysaccharides than the outer leaf-bases. Similar results were reported by Jaime et al. (2001), who used two methods for their assays to optimize the extraction procedure.

Unfortunately, none of these studies assayed the distribution and accumulation of the different FOS across leaf-bases during growth, only in mature harvested onions. Shiomi et al. (1997) studied the accumulation of different FOS and their metabolizing enzymes in three varieties of onion bulbs during growth. First, they identified two trisaccharides, three tetra-saccharides and four penta-saccharides, together with a mixture of hexa- and hepta-saccharides. Secondly, they assessed different FOS in whole onion bulb tissues of these three cultivars and noted that they increased from July 28 to August 26, then decreased, but to a higher level than that observed on June 26. They also noted that glucose increased in two cultivars, while sucrose and fructose increased in one. However, the general pattern of these variations was in agreement with the present study. The results showed that FOS increased from June to August, while sucrose and especially fructose remained low. This pattern was due to the mobilization of fructose and sucrose, which are the main substrates for the enzymes involved in FOS synthesis. The increase in FOS was due to their translocation from the aerial leaves to the bulb, indicating the beginning of maturity. The decrease observed during September was probably caused simultaneously by (i) the reduction of FOS biosynthesis and their exportation from the green leaves to the bulb tissues as the bulb reached maturity, (ii) the increase in the water content of the bulb tissues, resulting from higher water uptake during this period of drought stress. This physiological stress is well known to be the main factor inducing bulb dormancy. On the other hand, as illustrated in Figure 6, the balance observed between saccharides and FOS in the carbohydrate content during bulb formation is in agreement with the claim that fructans have osmoregulator function, providing osmotic adjustment during bulbing.

Conclusions

There is evidence that the contents of different FOS in onion bulb tissues during growth vary differently in the leaf-bases depending mainly on the growth stage and maturity. The high levels observed in August gave a clear demonstration that the accumulation of fructans occurs during this hot period, suggesting the end of bulbing and the possible harvesting stage. The results also showed that some FOS isomers accumulated more than others across the leaves, probably because their synthesis pathway was more favoured or they were degraded to a lesser extent during growth. This accumulation also seemed to be osmotically regulated, maintaining a balance between mono- and di-saccharides and FOS. Moreover, the carbohydrate content could be used as an effective tool for the prediction of the onset of bulb development and for the better prediction of maturity, which is considered one of the main factors of good storability in onion bulbs. Total carbohydrates could also be of value for estimating optimal maturity and harvest dates, to avoid dry matter losses during the last days of growth. Finally, further studies are recommended to assess FOS accumulation under different environmental conditions and in different cultivars, because these are key factors in bulbing and FOS accumulation.

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COMPARISON OF METHODS FOR INORGANIC TRACE ELEMENT ANALYSIS IN CROATIAN OLIVE OILS

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The aim of the present study was a comparison of the analytical methods optimized for the determination of trace elements in olive oils as a basis for further investigations, such as adulteration detection or geographical characterization.

Different types of sample preparation procedures prior to ICP-AES and GFAAS determinations were investigated: both open and closed vessel digestion in a steel bomb, as well as microwave-assisted digestion using a closed system, which was selected for further investigations. Recoveries for all elements in olive oil were > 95%. Good reproducibility (up to 10% RSD) was achieved for the measurements of the elements analysed. The sensitivity of the ICP-AES technique was sufficient for the determination of Ca, Fe, Mg and Zn (relative standard deviation approx. 2%). Furthermore, the proposed digestion method allowed the GFAAS determination of Al, Co, Cu, K and Ni in the concentration range of 0.1 to 1.5 μg/g, with relative standard deviations of approximately 3 to 10% for all samples.

Key words: GFAAS, ICP-AES, olive oil, trace elements, heavy metals

Introduction

The knowledge of the metal content in olive oils is important not only because of their nutritional value, but also for oil adulteration detection and the geographical characterization of the product, since this reflects the inorganic pattern of the soils where the olive trees grow.

Commonly used methods for the detection of olive oil adulteration are various chromatographic techniques (GC, RPLC-GC, GC-MS) (Buldini et al., 1997; Mandl et al., 1999) for the determination of organic components, and spectroscopic methods (Karadjova et al., 1998; Maurillo et al., 1999; Cordella et al., 2002) for the determination of the elemental composition, since the metal content is specific to each kind of edible oil (Juranović Ćindrić et al., 2007).

The determination of trace elements in oils is also important because of the metabolic role of some of the metals. Furthermore, some metal ions (Cu, Fe, Ni, Mn, Zn) influence the oxidative stability of edible oils (Karadjova et al., 1998). Copper, iron, lead and nickel are indicators for contamination during processing (Jacobs and Klevay, 1975; Maurillo et al., 1999) or via the environment (Allen et al., 1998; Multon, 1997; Pugh et al., 2002).

The most commonly used techniques for the determination of metals in oil samples are inductively coupled plasma atomic emission spectrometry (ICP-AES) and atomic absorption spectrometry (AAS) (Salvador et al., 1983; Turunen et al., 1995; Pomazal et al., 1999; Prohaska et al., 2000;). The main problems for the analysis of trace metal content are connected with the dissolution of fats and oils. Before analysing the edible oils by spectrometric techniques it is necessary to bring the elements into an aqueous solution. Among the different methods of sample preparation, the wet and dry digestion methods are the most suitable procedures for a total dissolution of the elements. However, many wet or dry digestion methods are not recommended for use with high-fat material because of the associated safety hazards. The measurement of a direct dilution of the oil sample with a suitable organic solvent, followed by direct aspiration into an atomic absorption flame or an ICP-plasma is sometimes not sufficiently sensitive. One disadvantage of this method is that oil droplets larger than 10 μm in size may pass through the flame (flame-AAS) or the plasma (ICP-AES) without being atomized (Guardia and Vidal, 1984). One way of avoiding these problems is to use emulsions. In an emulsion the oil is incorporated in the aqueous phase and can be directly introduced into the burner. The limitations of such a method are the instability of emulsions, the selection of a suitable surfactant, the proportions of the phases, the preparation procedure, the nature of the compounds present in the matrix and the fact that in this case aqueous standard solutions cannot be used for calibration (Guardia and Vidal, 1984; Goncalves et al., 1998; Maurillo et al., 1999).

Trace element extractions have also been used as separation procedures. These methods suffer from disadvantages such as high time consumption, possible loss of volatile metal species, contamination by digestion or chelating processes, or non-quantitative recovery (Price et al., 1970).

An optional method of sample preparation is microwave-enhanced dissolution, which is fast, efficient and reproducible (Maurillo et al., 1999; Richter et al., 2001).

In the present study, ICP-AES and graphite furnace-AAS (GFAAS) determinations of the elements in olive oil were optimized. Several methods were compared for the analysis of olive oil as a representative of edible oils, and differences between the types of dissolution procedures applied are discussed. The methods involve various sample preparation techniques, namely open-vessel and closed-vessel digestion in a steel bomb, as well as microwave-assisted digestion in a closed system. The closed-vessel dissolution procedure is a suitable procedure prior to the ICP-AES and GFAAS determination of metals. Both simultaneous and sequential analyses are reported. These methods were evaluated by the application of the standard addition method and by recovery experiments.

Materials and methods

Chemicals and glassware

For the experimental work nitric acid (65 w%, analytical grade), hydrogen peroxide (30 w%, analytical grade) (Merck, Darmstadt, Germany) and both single element and multielement standards (ICP Multielement Standard IV, Merck, Darmstadt, Germany) were applied. Standard stock solutions were used for the preparation of aqueous standard solutions after appropriate dilution. All olive oil samples were products available on the Croatian market. All glassware was cleaned with 7 M nitric acid prior to use.

Apparatus

ICP-AES was performed using an ARL 3580 ICP spectrometer (ARL, Ecublens, Switzerland) working in simultaneous-sequential mode, equipped with an HF-generator (Henry, 27.12 MHz), and an RF power supply (1200 W). The spectrometer uses a Paschen-Runge mounting equipped with a 1080 lines/mm grating, a Babington-type nebulizer (ARL MDSN), a Fassel type torch and a computer (DEC 316 sx). The applied gas flows (L/min) were outer: 12, intermediate: 0.8 and aerosol carrier gas: 1. The observation height was 15 mm above the coil.

Very low concentrations of the elements were measured with a Perkin Elmer PE 4100 ZL atomic absorption spectrometer equipped with a Zeeman background correction using end-capped graphite tubes with L'vov platforms. All instrument-operating parameters were given in a previous publication (Juranovic et al., 2003).

For the microwave-assisted digestion of the samples a high performance microwave digestion unit MLS-1200 MEGA with a EM-30 unit (Milestone GmbH) was applied.

Sample preparation for ICP-AES and GFAAS

The samples of olive oils were weighed and subsequently digested using three different procedures: open-vessel or closed-vessel wet digestion under pressure in a steel bomb, and microwave-assisted digestion. In every case the samples were treated with a mixture of nitric acid and hydrogen peroxide. After digestion clear solutions were obtained. For each series of digestions a reagent blank was prepared. Subsequently the samples were analysed by ICP-AES and/or GFAAS.

For the open-vessel digestion 1 g of oil was weighed into a glass vessel. After the addition of 5 mL of conc. HNO_3 plus 0.5 mL of H_2O_2 (30 w%) the samples were heated for approx. 12 hours on a heating plate at approx. 120°C. During digestion the addition of reagents was repeated twice. After this procedure all the samples were transferred into 10 or 20 mL volumetric flasks and diluted to volume with 1 M HNO_3.

For the closed-vessel digestion a steel bomb equipped with a PTFE inlet was used. To each aliquot of 0.25 g oil, 5 mL of conc. HNO_3 plus 15 drops of H_2O_2 was added and the mixture was then heated to 100°C for approx. 12 hours. After digestion the samples were transferred into 10 mL volumetric flasks and diluted to volume with 1 M HNO_3 for measurement.

For the microwave digestion procedure 0.5 g oil was placed into the PTFE-digestion vessels and 4 mL of conc. HNO_3 plus 2 mL H_2O_2 was added. A rotating turntable was used to ensure the homogeneous distribution of the microwave radiation. A sensor was used to measure the temperature in one vessel throughout the digestion process. The oil samples were digested according to the following optimized program (power in W/time in min): 250/2, 0/1, 250/2, 600/1, 400/5, ventilation 3.0 min. The internal temperature was limited to 240°C. After cooling, all the digests were transferred into 10 mL volumetric flasks and diluted to volume with 1 M HNO_3.

Calibration procedure

For both the ICP-AES and the GFAAS method, measurements were accomplished by calibration with matrix-adjusted mixed standards prepared in HNO_3 (1 M; dilution 1:14). All calibration curves were based on five standards, including the blank. Standard solutions were prepared by diluting a 1000 mg/L multielement solution. The concentration ranges for the elements were: 5 to 100 mg/L for Al, Cd, Co, Cr, Cu, Fe, Mg, Mn, Mo, Ni, Pb, Ti, V and Zn, and 500 to 1000 mg/L for Ca, K and P. The calibration ranges were modified according to the expected concentrations of the elements of interest and depending on the technique applied (ICP-AES or GFAAS).

Spiking procedures

The olive oil samples were spiked by adding aqueous standard solutions to the set of samples prepared as previously described for pumpkin seed oil (Juranovic et al., 2003). Approx. 0.5 g olive oil was transferred into digestion flasks; 1 mL of the respective standard solution (c=100 mg/L) was added. After addition of a mixture of nitric acid and hydrogen peroxide the samples were digested in a microwave unit. The clear digests were transferred into 10 mL flasks and filled to volume with 1 M HNO_3. The resulting concentration of each element was 10 mg/L. All spiked samples were prepared in triplicate (a, b and c) and measured by ICP-AES and GFAAS.

ICP-AES determination

Prior to analysis, line selections were performed (Juranovic et al., 2003). The background correction positions chosen were +0.14 nm from the peak maximum. The integration time used was 5 s. Spectral interferences were checked before the ICP-AES measurements. For the determination of K and P, sequential lines were selected, while for all the other elements a simultaneous program was set up.

In order to check the accuracy of the method, recovery experiments were carried out by adding 10 mg/L for each element.

GFAAS determination

The wavelengths, as well as the pretreatment and atomization temperatures used during the analyses, are listed in previous papers (Zeiner et al., 2005; Juranović Ćindrić et al., 2007;). Pyrolytically coated graphite tubes were used.

The same sample solutions, obtained by microwave-assisted digestion, were used for both ICP-AES and GFAAS measurements. Dilution of the samples was necessary for the GFAAS determination to confirm the ICP-AES results obtained for the major elements, because of the higher sensitivity of the former technique.

Limits of detection (LOD)

The limits of detection for the ICP-AES analyses were determined separately for each analytical sample by measuring an appropriate reagent blank solution eleven times and the matrix-adjusted standard solution three times. The LODs were calculated according to Boumans (1987) as the concentration equivalent to three times the standard deviation (3σ) of the signal of the blank solution. All standard deviations are based on measurements in triplicate.

Results and discussion

All the methods of digestion tested led to clear digest solutions. The metal concentrations for the elements of interest are listed in Table 1 for the ICP-AES measurements, and in Table 2 for the GFAAS determinations.

After digestion with a mixture of nitric acid and hydrogen peroxide the clear digests of fourteen olive oil samples were measured by ICP-AES and GFAAS. Slightly different results were obtained depending on the digestion method used. The results obtained after open vessel-digestion were lower and varied significantly according to the *t*-test. They exhibited poor reproducibility compared to the closed-vessel digestion (steel bomb or microwave unit) method. The disadvantages of the open-vessel digestion for the given purpose are losses of volatile metals and the incomplete dissolution of the oil matrix, causing matrix effects. Compared to the closed-vessel systems, samples treated in open vessels are exposed only to atmospheric pressure, compared to pressures of up to 40 bar when using a microwave digestion oven.

Good reproducibility was found for the determination of all elements for measurements using ICP-AES (approx. 2% RSD), while the reproducibility was approx. 3 to 10% RSD for the elements measured with the GFAAS technique. Differences between the results obtained for the two closed-system digestions were very small (Table 1), and according to the *t*-test none were statistically significant. This indicates that in both cases the digestion of the olive oil was complete. In the first case microwave radiation was applied. The microwaves destroy the oil matrix very rapidly, in addition to the influence of the elevated temperature, the higher pressure, and the oxidative effects of the acids used.

The sensitivity of the ICP-AES technique was sufficiently high for the determination of Ca, Fe, Mg and Zn. Contents of 2 to 3 μg/g for Ca, Mg and Zn were found, and 15 μg/g for Fe in olive oil. The concentrations of Al, Cu, Co, Cr, K, Ni, Mn and Pb were lower (< 0.1 μg/g). For the measurements performed by ICP-AES after open- or closed-vessel digestion the relative standard deviations (RSD) were approx. 5–10% except for Ni, where RSD% was ≥ 16.8 (Table 1). Therefore, Ni was measured by GFAAS (Table 2).

The concentrations of Al, Co, Cu, K and Ni were in the range of 0.1 to 1.5 μg/g, which was close to the LODs for measurements by ICP-AES. Therefore, these elements were measured by GFAAS. The results obtained were 0.15 μg/g for Al, 0.12 μg/g for Co, 0.14 μg/g for K, 0.55 μg/g for Cu and 1.5 μg/g for Ni (Table 2). The concentrations of Mn, Cr and Pb were even lower than the LODs of the GFAAS determination (< 1 ng/g). For the measurements performed by GFAAS after open- or closed-vessel digestion the relative standard deviations (RSD) were approx. 5–12% (Table 2).

The recoveries for all the elements investigated were in the range of 95 to 110% with RSDs lower than 10%. Further improvements in the performance of the method are limited by the sample amount when applying microwave-assisted digestion and by the reagent volumes.

Comparing ICP-AES and GFAAS it can be stated that the sample need is higher for ICP-AES (1–2 mL) than for GFAAS (0.2–0.5 mL). The limits of detection achievable for ICP-AES are higher than those obtained by GFAAS. The time per sample required using ICP-AES is much lower than for GFAAS. Further, with GFAAS the element in question can be enriched by pipetting the sample into the graphite tube several times. Thus GFAAS is appropriate for the determination of elements present in very low concentrations. The multi-elemental method ICP-AES is preferably used for the analysis of the minor and major elements of oils and especially in cases when more than one element is to be quantified.

Table 1

Determination of elements in olive oil by ICP-AES after microwave digestion, open-vessel digestion and digestion in a steel bomb. Results are expressed in μg/g oil

Olive oil digested in	ICP-AES					
	Ca	Mg	Fe	Zn	Ni	Al, Cu, Co, Cr, K, Mn, Pb
Microwave oven	2.0	3.5	15.4	3.4	1.1	< LOD[a]
RSD [%]	4.9	5.2	7.1	6.5	16.8	
Steel bomb	2.1	3.3	15.2	3.5	0.8	< LOD[a]
RSD [%]	5.7	6.2	7.8	5.9	18.0	
Open vessel	1.1	2.3	11.6	3.4		
RSD [%]	7.8	8.8	6.9	6.4	< LOD[a]	< LOD[a]

[a] LODs <0.03 μg/g for Mn, 0.1 μg/g for Ni and Pb, 0.2 μg/g for Co, Cu and K, >0.7 μg/g for Al and Cr.

Table 2

Determination of elements in olive oil by GF-AAS after microwave digestion, open-vessel digestion and digestion in a steel bomb. Results are expressed in μg/g oil

Olive oil digested in	GFAAS					
	Al	Cu	K	Co	Ni	Mn, Cr, Pb
Microwave oven	0.15	0.55	0.14	0.12	1.5	< LOD[a]
RSD [%]	8.5	10.0	9.5	9.1	8.0	
Steel bomb	0.13	0.57	0.15	0.09	1.3	< LOD[a]
RSD [%]	8.6	9.5	9.8	9.5	7.5	
Open vessel	< LOD[a]	0.22	0.05	< LOD[a]	0.2	< LOD[a]
RSD [%]		12.0	11.5		8.8	

[a] LODs <1 ng/g for Al, Co, Cr, Mn and Pb

Conclusions

It can be stated that among the digestion methods investigated for the elemental analysis of olive oils the microwave-assisted closed-vessel digestion method leads to the best results for all the elements of interest. A microwave-assisted decomposition procedure in closed vessels was developed using a mixture of nitric acid and hydrogen peroxide. Thereby the organic matrix of the samples was destroyed effectively. The procedure is simple, reproducible and relatively fast and no significant loss of the analysed elements was observed during the digestion step. The low achievable LODs enable the determination by ICP-AES of even very low concentrations of most elements of interest. Recovery for all elements was > 95%.

The ICP-AES method developed in this work permits the determination of Ca, Fe, Mg, Na and Zn in olive oil. Elements present in very small amounts can be measured by GFAAS in the same sample digest.

This analytical method represents a good basis for further investigations on olive oils, such as adulteration detection (Juranović Ćindrić et al., 2007) and geographical characterization (Zeiner et al., 2005). The differences in the inorganic patterns of oils from various regions in Croatia can be attributed to the metal composition of the soils where the plants were grown and to the technique of oil production.

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INFLUENCE OF AMMONIUM CHLORIDE ON GROWTH AND UPTAKE OF Fe, Cu, Zn AND B IN COTTON GROWN IN ALKALINE SOIL

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A pot culture experiment was conducted to study the influence of NH_4Cl (AnalaR grade or commercial fertilizer) on soil pH and on the growth, yield and nutrient uptake of cotton (cv. NIAB-Karishma) grown in alkaline soil. The experiment was carried out in a net-house under natural conditions. The soil used was clayey loam with pH 8.61, and ammonium chloride either from commercial fertilizer or of AnalaR grade (both containing up to 25% N) was applied in three split doses, after germination (10 days), at the vegetative stage (40 days) and at the flowering stage (80 days) @ 6, 12 and 18 kg ha^{-1}. The application of NH_4Cl decreased the soil pH and increased the plant height and cotton yield $plant^{-1}$. Plants treated with NH_4Cl AnalaR grade produced higher yields as compared to NH_4Cl commercial fertilizer. The uptake of micronutrients such as Fe, Cu, Zn and B was enhanced by NH_4Cl application in both the stem and leaves of cotton. However, AnalaR grade NH_4Cl proved more effective than NH_4Cl commercial fertilizer in all cases.

Key words: alkaline soil, ammonium chloride, cotton, nutrient uptake

Introduction

Irrigation water usually contains bicarbonate (HCO_3^-) and carbonate (CO_3^{2-}) as well as sodium, potassium, calcium and magnesium salts. The accumulated sodium carbonate and bicarbonate may increase the pH of soils up to values as high as 10. This increase in pH may cause nutritional deficiencies in the crop because high soil pH decreases the availability of nutrients (Millar, 1995). Micronutrients become scarce in alkaline soils as compared to acidic and neutral soils (Russell and Russell, 1973; Ashraf and Sarwar, 2002).

Cotton (*Gossypium hirsutum* L.) has the ability to grow well on a wide range of soil types, its main requirement being an adequate root range. It tolerates a fairly wide range of soil acidity and alkalinity and is known to withstand a pH range of 5 to 9 in many cases (Munro, 1987). Pakistan occupies

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the fifth position in the world in respect of cotton production. It is the major source of foreign exchange through the export of raw cotton, cotton yarn and piece goods. The cotton belt of Pakistan lies in the middle of the Indus valley in the Punjab and Sind provinces of Pakistan. The average cotton yield in Pakistan is 1583 kg ha^{-1}, which is unfortunately far less than in the advanced cotton-growing countries of the world (Govt. of Pakistan, 2005). This low production is due to many factors, the most important being soil salinity and alkalinity, which reduces crop production by decreasing the nutrient uptake necessary for plant growth.

One of the ammonium compounds being investigated to decrease soil pH is ammonium chloride. Fertilizer grade ammonium chloride usually contains 25% nitrogen, which is higher than that of ammonium sulphate. Although ammonium chloride is best known for its use in rice, it is also suitable for a variety of other crops including barley, wheat, maize, sorghum, sugarcane and fibre crops like cotton. Moreover, ammonium chloride is acid-forming and this effect can be used to decrease the pH of alkaline soils, but its high chlorine content will limit its use to tolerant crops (Tisdale et al., 1985; Kamal et al., 2003). Ashraf et al. (2005) reported that the application of ammonium chloride increased the nitrate reductase activity, growth and nutrient uptake of Na^+, K^+, Ca^{2+} and Cl^- but not that of P in cotton grown in alkaline soils. Its use is beneficial in alkaline soils to increase the availability of the nutrients necessary for proper plant growth and productivity (Jeong and Lee, 1996). Brar et al. (1993) and Khalil (1998) also showed that ammonium fertilizers help to obtain optimum crop yields on alkaline soils.

The micronutrients Fe, Cu, Zn and B are necessary for optimum plant growth because they are either an integral part of enzymes, coenzymes, protein, etc. or have key roles in metabolic activities (Brady and Weil, 1999). Fe plays a role in many plant enzyme systems, is a component of certain plant proteins, and is involved in energy transfers, reduction reactions and as a catalyst. It also participates in protein synthesis and root tip meristem growth (Jones et al., 1991). Copper is involved in many of the plant's biochemical pathways. It is a constituent of plastocyanin and participates in the protein and carbohydrate metabolism and in nitrogen fixation. It is a component of the enzymes involved in the respiration process of plants (Tagliavini et al., 1995). Similarly Zn is a component of chlorophyll and a cofactor in many enzyme reactions. It is involved in the redox reactions of the photosynthetic electron transport system. In photolysis it acts as a bridge for ATP and for enzymes (Singh and Raj, 1994; Moreno et al., 1996). Boron is involved in one of the bases for RNA (uracil) and in cellular activities (division, differentiation, maturation, respiration and growth). It is associated with both pollen germination and growth and with calcium uptake, but is fairly immobile in plants (Tisdale et al., 1985; Jones et al., 1991).

The availability of micronutrients and the metabolic activities of plants growing on alkaline soils are adversely affected by alkalinity, which ultimately reduces plant growth and yield. Therefore, keeping in view the importance of micronutrients, cotton growth and yield, and the acid reaction of NH_4Cl, the present study was conducted to examine the effect of ammonium chloride on soil pH, growth and micronutrient uptake in cotton grown in alkaline soil.

Materials and methods

An experiment was conducted in pots to study the effect of ammonium chloride on the pH and nutrient uptake of cotton. Two sources, i.e. commercial fertilizer (Grow Plan, Rockhampton, Australia) and AnalaR grade (Merck), were used in the present study. Seeds of cotton (*Gossypium hirsutum* L.) variety NIAB-Karishma were obtained from the Entomology Division of NIAB, Faisalabad, Pakistan. The NPK fertilizers were applied as 120, 70 and 70 kg N, P_2O_5 and K_2O ha^{-1}, respectively. The soil was analysed for texture, EC, pH and saturation percentage before use (USDA, 1962). It was a clayey loam with pH 8.61, EC 1.61 dS m^{-1} and a saturation percentage of 34%. The experiment consisted of seven treatments including a control, each with five replicates, laid out in a completely randomized design (CRD) with a two-factorial arrangement. The ammonium chloride (NH_4Cl) treatments are listed in Table 1. The meteorological data for the cotton growing season of 2004 are summarized in Figure 1. During the experiment the maximum temperature ranged from 34.3–43.3°C, the minimum temperature from 19.7–9.3°C, the rainfall from 7.4–79.2 mm, the relative humidity from 25.5–49.0% and the radiation from 7–9 $MJ/m^2/day$ (Fig. 1A, B, C, D).

Table 1

Treatment details

Treatment code	Description	Rates
T_o	Without ammonium chloride (control)	–
T_1	NH_4Cl as commercial fertilizer	6 kg ha^{-1}
T_2	NH_4Cl as commercial fertilizer	12 kg ha^{-1}
T_3	NH_4Cl as commercial fertilizer	18 kg ha^{-1}
T_4	NH_4Cl AnalaR grade	6 kg ha^{-1}
T_5	NH_4Cl AnalaR grade	12 kg ha^{-1}
T_6	NH_4Cl AnalaR grade	18 kg ha^{-1}

Ammonium chloride was added in three split doses, after germination (10 days), at the vegetative stage (40 days) and at the flowering stage (80 days), and the growth data and yield parameters were recorded. At physiological maturity the aboveground portion of the plant was harvested and the leaves and stem were separated and dried at 65±5°C in a forced air oven (OV-190F, GenLab Widnes, England). The dried samples were ground with a grinder (Dietz, Germany), followed by digestion in H_2SO_4-H_2O_2 according to Wolf (1982). After filtration through Whatman-40, aliquots were used for the spectrophotometric (6300, Jenway, England) determination of B according to Wolf (1971), while Cu, Zn and Fe were determined from the stem and leaves of cotton using an atomic absorption spectrophotometer (Perkin Elmer, 5100, USA). The data were subjected to statistical analysis using the Analysis of Variance technique and Duncan's Multiple Range Test (DMRT) was applied to compare the means (Steel and Torrie, 1980).

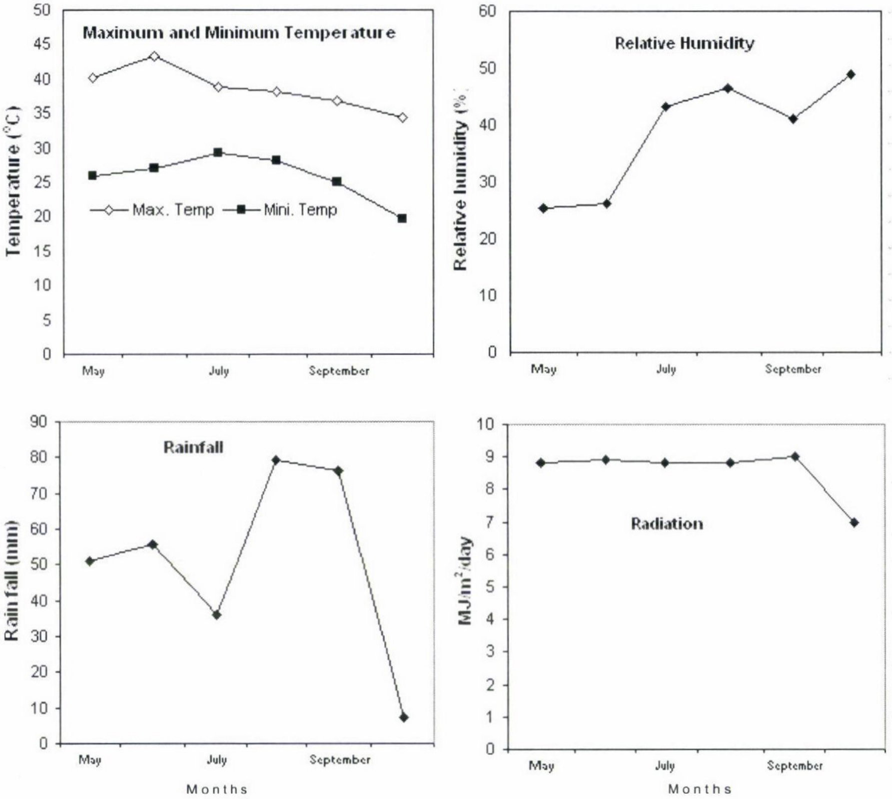

Fig. 1. Meteorological data during the experimentation

Results

Soil analysis

The results of soil analysis showed that the application of ammonium chloride to clayey loam soil decreased the pH of the soil from 8.61 to 7.27 in the case of commercial fertilizer and to 7.12 for AnalaR grade at the highest NH_4Cl level (Fig. 2A). A slight decrease in soil EC was also observed (Fig. 2B).

Growth and yield

The shoot length of cotton plants was significantly influenced by the different levels of ammonium chloride, but non-significantly affected by the source of ammonium chloride and the source × level interaction (Table 2). Shoot length increased with an increase in NH_4Cl level for both sources (Fig. 3A). The highest shoot length was recorded in plants treated with 18 kg NH_4Cl ha^{-1}, while the minimum shoot length was observed for the control. Neither of the sources was superior to the other.

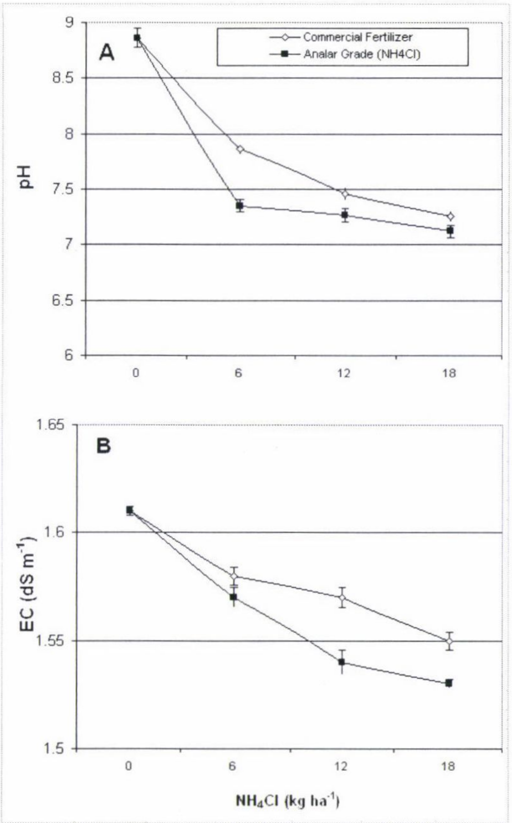

Fig. 2. Influence of commercial fertilizer and AnalaR grade NH_4Cl on soil pH (A) and soil EC (B)

Table 2
ANOVA. Mean squares for different parameters

Parameters	Source of variation			
	NH_4Cl sources	Treatments	Sources × treatment	Error
Degree of freedom	1	3	3	16
Shoot length	6.00^{NS}	28.67**	0.72^{NS}	7.35
Number of bolls plant^{-1}	1.50^{NS}	4.50^{NS}	0.28^{NS}	1.52
Cotton yield plant^{-1}	9.34**	48.27**	1.70*	0.44
Stem: iron (Fe)	522.67**	4093.83**	81.44**	12.46
Leaves: iron (Fe)	5.04^{NS}	3143.82**	4.60^{NS}	12.08
Stem: copper (Cu)	0.04^{NS}	28.82**	0.60^{NS}	0.21
Leaves: copper (Cu)	15.04**	35.38**	3.26**	0.25
Stem: zinc (Zn)	273.38**	858.38**	68.49**	3.88
Leaves: zinc (Zn)	640.67**	1497.00**	124.78**	7.58
Stem: boron (B)	28.17**	33.00*	6.06**	1.25
Leaves: boron (B)	330.04*	585.93**	100.71^{NS}	50.00

NS = Non-significant, *, ** = Significant at the $P \leq 0.05$, $P \leq 0.01$ level, respectively

The maximum number of bolls (10 plant^{-1}) was recorded at 18 kg NH_4Cl ha^{-1} AnalaR grade and the minimum (7.67 plant^{-1}) in the control (Fig. 3B). The number of bolls per plant did not differ significantly in the various treatments (Table 2). However, a slight increase was observed with an increase in NH_4Cl level for both the sources (Table 2). The cotton yield per plant increased with an increase in NH_4Cl level for both sources (Fig. 3C). Plants treated with 18 kg NH_4Cl ha^{-1} of AnalaR grade had maximum cotton yield (26.42 g plant^{-1}), while the lowest yield (19.13 g plant^{-1}) was found in the control. A comparison between the NH_4Cl sources showed that AnalaR grade was more successful in enhancing the cotton yield than commercial fertilizer.

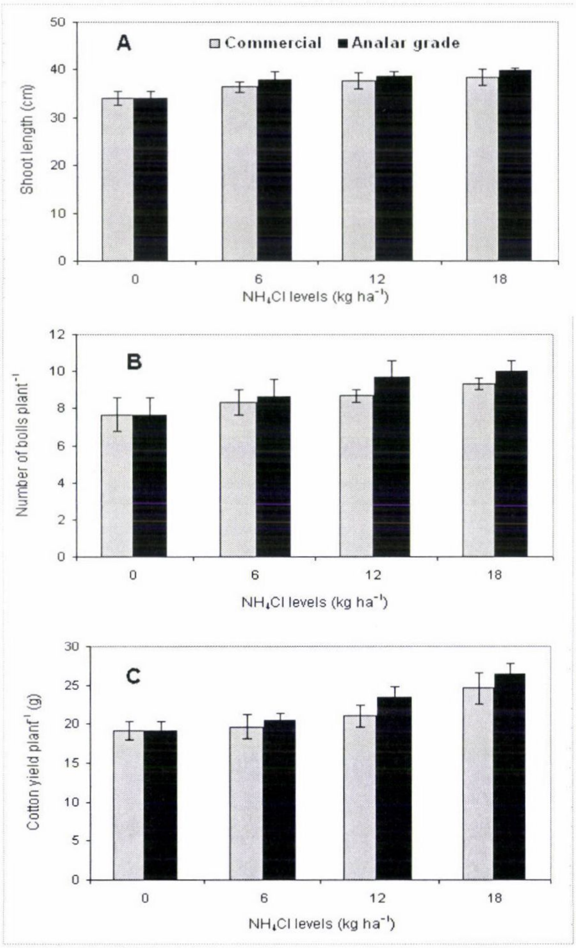

Fig. 3. Effect of different levels of commercial fertilizer and AnalaR NH_4Cl on shoot length (A), number of bolls plant^{-1} (B) and cotton yield plant^{-1} (C) of cotton variety NIAB-Karishima

Micronutrients

The iron (Fe) content in the cotton stem was significantly influenced by the application of NH_4Cl (Table 2), increasing with an increase in the NH_4Cl level (Fig. 4A). The highest stem Fe content (1.98 $\mu mol\ g^{-1}$ DW) was observed at 18 kg NH_4Cl ha^{-1} AnalaR grade, and the lowest (0.786 $\mu mol\ g^{-1}$ DW) in plants grown without NH_4Cl (control). AnalaR grade NH_4Cl treatment was more effective in enhancing the Fe content in the stem than commercial fertilizer (Table 2; Fig. 4A). Similarly the Fe content in the leaves increased with the application of NH_4Cl (Table 2; Fig. 4B), the highest Fe contents (2.51 and 2.52 $\mu mol\ g^{-1}$ DW) being observed for the highest level of NH_4Cl from both the sources and the lowest (1.61 $\mu mol\ g^{-1}$ DW) in the control. Both the sources had a similar effect on leaf Fe content (Table 2).

The Cu content in the stem increased with an increase in the NH_4Cl concentration in the soil. The action of both NH_4Cl sources on stem Cu content was similar (Table 2). The highest stem Cu contents (0.115 and 0.131 $\mu mol\ g^{-1}$ DW) were obtained at the highest level of NH_4Cl and the lowest (0.0724 $\mu mol\ g^{-1}$ DW) in plants growing without NH_4Cl (Fig. 4C). The source had no significant effect on stem Cu content (Table 2). In the leaves, NH_4Cl of AnalaR grade gave a significantly higher Cu content than commercial fertilizer (Table 2). The highest leaf Cu content (0.183 $\mu mol\ g^{-1}$ DW) was obtained at 18 kg NH_4Cl ha^{-1} AnalaR grade and the lowest (0.047 $\mu mol\ g^{-1}$ DW) in the control. An increasing trend in Cu was observed for both types of NH_4Cl, but AnalaR grade NH_4Cl performed better than commercial fertilizer (Table 2; Fig. 4D).

The Zn content in the stem of cotton plants significantly increased with an increase in NH_4Cl level (Table 2). The two NH_4Cl sources differed significantly, the AnalaR grade performing better than commercial fertilizer. In the stem the highest Zn content (0.728 $\mu mol\ g^{-1}$ DW) was recorded at 18 kg NH_4Cl ha^{-1} of AnalaR grade and the lowest (0.2 $\mu mol\ g^{-1}$ DW) for the control (Fig. 5A). The Zn content recorded for all three levels of NH_4Cl commercial fertilizer was comparatively less than that obtained with AnalaR grade (Fig. 5A). Similar results were obtained for cotton leaves, where the highest Zn contents (1.092 $\mu mol\ g^{-1}$ DW) were also recorded at 18 kg NH_4Cl ha^{-1} of AnalaR grade and the lowest (0.425 $\mu mol\ g^{-1}$ DW) for the control (Fig. 5B). The two NH_4Cl sources differed significantly for leaf Zn contents (Table 2) and NH_4Cl of AnalaR grade was more effective in enhancing the Zn uptake than commercial fertilizer. The Zn content in the leaves increased with an increase in the NH_4Cl concentration in the soil, and maximum Zn contents were recorded at the highest level of NH_4Cl for both sources (Fig. 5B).

The boron (B) content in the cotton stem decreased from the control value (1.391 $\mu mol\ g^{-1}$ DW) to 0.936 and 1.027 $\mu mol\ g^{-1}$ DW in commercial and AnalaR grade, respectively, at a rate of 6 kg NH_4Cl ha^{-1}. At 12 and 18 kg NH_4Cl ha^{-1} commercial fertilizer the B content increased, but the values were lower than in the control. However, a steady increase in stem B contents over the control was observed at 12 and 18 kg NH_4Cl of AnalaR grade (Fig. 5C), the maximum B content (1.664 μmol g^{-1} DW) being recorded at 18 kg NH_4Cl ha^{-1} followed by 1.445 μmol g^{-1} DW at 12 kg NH_4Cl ha^{-1}. In the leaves the B content increased with an increase in NH_4Cl level for both the sources (Fig. 5D). The AnalaR grade source performed significantly better than commercial fertilizer (Table 2). In the leaves the maximum B content (11.727 μmol g^{-1} DW) was found at a rate of 6 kg NH_4Cl ha^{-1} (AnalaR grade) compared with the minimum (9.273 μmol g^{-1} DW) in the control (Fig. 5D).

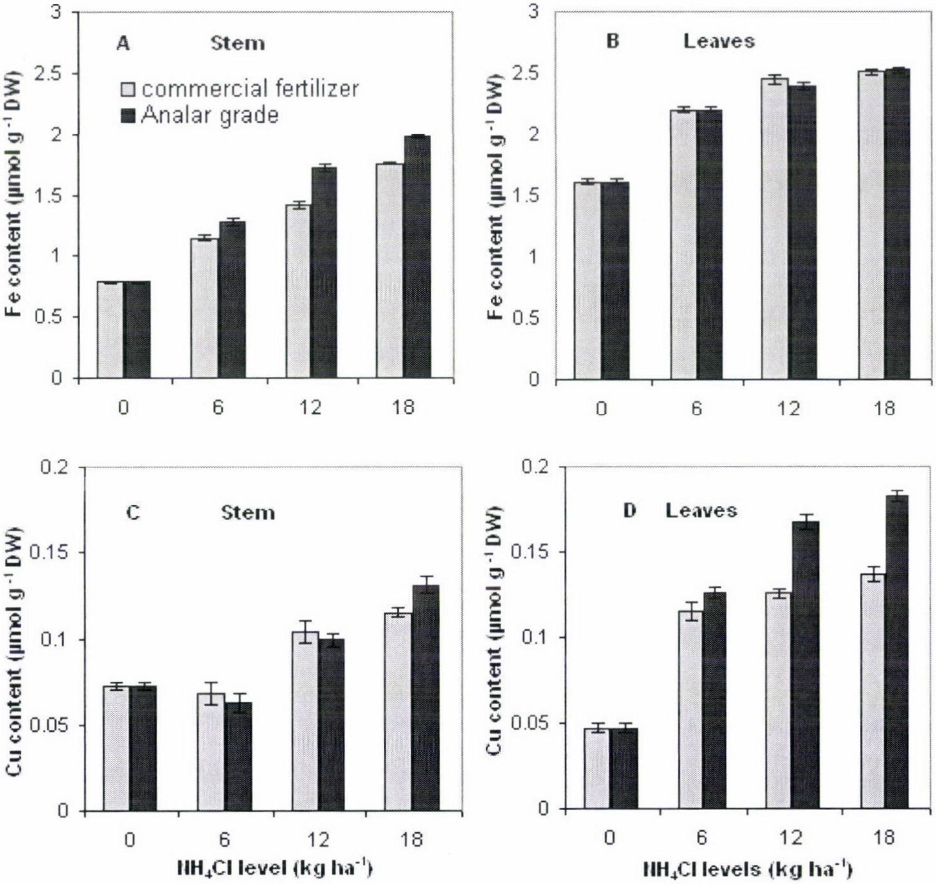

Fig. 4. Effect of different levels of commercial fertilizer and AnalaR NH_4Cl on the iron content in stem (A) and leaves (B), and the copper content in stem (C) and leaves (D) of cotton variety NIAB-Karishima

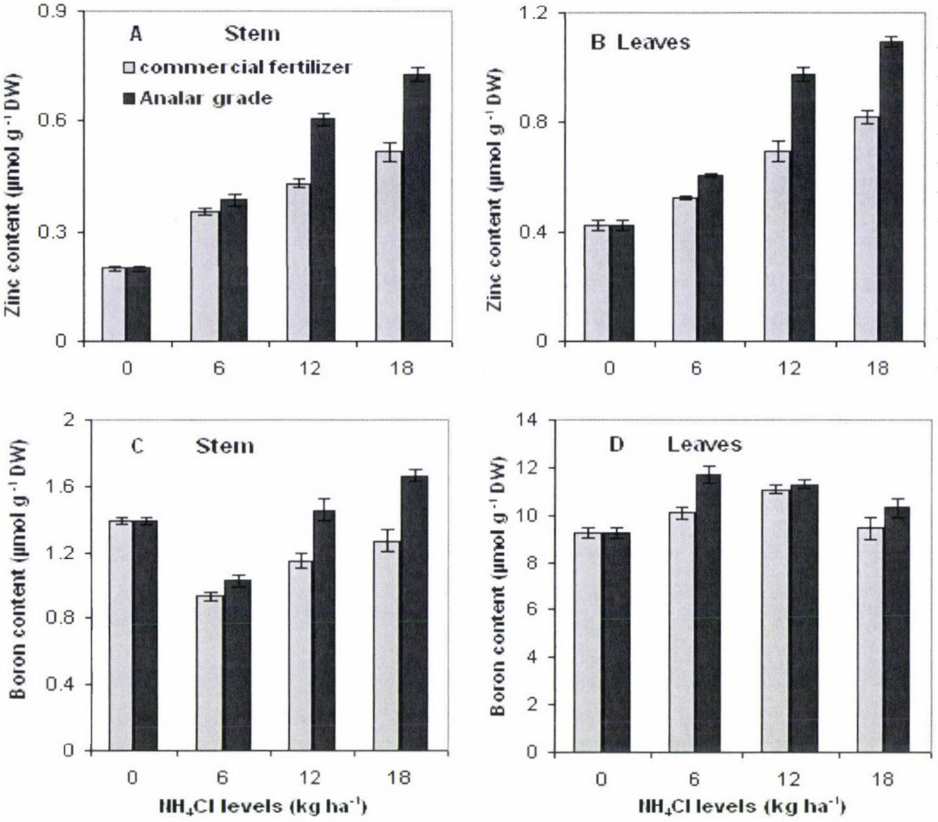

Fig. 5. Effect of different levels of commercial fertilizer and AnalaR NH_4Cl on the zinc content in stem (A) and leaves (B), and the boron content in stem (C) and leaves (D) of cotton variety NIAB-Karishima

Discussion

Ammonium chloride is an acid-forming compound, which plays a key role in decreasing the pH of the soil, thus increasing the availability of various nutrients and ultimately increasing growth and yield (Tisdale et al., 1985; Ashraf et al., 2005). The results of the present study confirmed the above statement, as the application of NH_4Cl reduced the pH of the soil (Fig. 2A). Although both the sources were effective in reducing the soil pH, AnalaR grade gave better results than commercial fertilizer.

The increase in plant height with an increase in NH_4Cl application indicated the positive effect of NH_4Cl on plant growth. The increase in shoot length was more pronounced for NH_4Cl AnalaR grade treatments compared to

NH_4Cl commercial fertilizer (Fig. 3 A). This greater effectiveness may be due to the lower concentration of available NH_4Cl in the latter due to the presence of microorganisms. Several reports (Brar et al., 1993; Barsoom, 1996) also indicated that low nitrogen supplies increased plant height. The number of bolls plant^{-1} and yield plant^{-1} also increased due to NH_4Cl application. The increase in the number of bolls was non-significant (Fig. 3B), while yield plant^{-1} (Fig. 3C) was significantly enhanced. This might be due to the size of the boll, which was much larger for treated plants as compared to the control. The highest yield was recorded at the highest level of NH_4Cl AnalaR grade (18 kg ha^{-1}), which may be due to the higher supply of N and the greater availability of nutrients due to a decrease in soil pH (Jeong and Lee, 1996). As the yield plant^{-1} was not as high as desired, it may be further increased by fulfilling the N requirement of the crop by increasing the dose of NH_4Cl or by applying some other form of N, such as urea, in addition to NH_4Cl. The results obtained with various N sources, such as urea, $(NH_4)_2SO_4$, NH_4NO_3, etc., have been reported by various research workers (Brar et al., 1993; Khalil, 1998).

Micronutrients such as Fe, Cu, Zn and B are necessary for optimum plant growth. The application of NH_4Cl increased the uptake of these micronutrients by decreasing soil pH. Similar results were observed by Tagliavini et al. (1995) for $(NH_4)_2SO_4$. The increase in the availability of micronutrients was correlated with higher growth and yield. The Fe content in both the stem and leaves of cotton plants was increased by both sources of NH_4Cl. Plants treated with NH_4Cl AnalaR grade had significantly higher stem Fe contents (Fig. 4A), while in the leaves both sources showed similar results at all levels (Fig. 4B). This indicated the higher effectiveness of AnalaR grade over commercial fertilizer. The Fe requirements of the plants remained below the toxic level. Fe is an important micronutrient because it is a component of many enzymes, such as cytochrome peroxidases and catalases. It is also a major constituent of the protein ferredoxin necessary for nitrate and sulphate reduction and for nitrogen assimilation (Khan et al., 1990; Briat et al., 1995). It acts as a co-factor in many enzyme systems necessary for the synthesis of chlorophyll (Han and Xu, 1996; Ashraf et al., 2003). Fe is also involved in protein synthesis and the growth of the root-tip meristem in plants (Jones et al., 1991). In soils with high pH, the availability of Fe becomes deficient (Singh and Raj, 1994; Tang et al., 1995). As NH_4Cl decreased the soil pH, the uptake of Fe by the plants was facilitated, resulting in optimal metabolic activity, and improved plant growth and yield.

The effect of NH_4Cl on copper (Cu) uptake was similar to that of Fe, as it was also enhanced with an increase in NH_4Cl level (Fig. 4). However, NH_4Cl AnalaR grade was more effective than commercial fertilizer in cotton leaves (Fig. 4D), while in the stems (Fig. 4C) both sources had the same effect. The availability of Cu decreases greatly at high pH (Singh and Raj, 1994; Tang et al., 1995). As NH_4Cl decreased the soil pH, the availability of Cu to the plants increased. The importance of Cu in plants can be clearly understood by its various functions. It plays a key role in the synthesis of plastocyanin, proteins and chloroplast (Han and Xu, 1996). It also serves as a component of plastocyanin in the electron transport chain (ETC) linking photosystems I and II. Cu participates in the protein and carbohydrate metabolism and in N assimilation. It is also a cofactor of several enzymes such as cytochrome oxidase, ascorbic acid oxidase and polyphenol oxidase (Moreno et al., 1996). Cu is also involved in the desaturation and hydroxylation of fatty acids (Jones et al., 1991). Thus an increase in Cu uptake may have enhanced these processes, resulting in increased growth and yield.

The Zn uptake was also enhanced by an increase in the NH_4Cl level. Its content in both stem and leaves was higher for NH_4Cl AnalaR grade than for commercial fertilizer (Fig. 5A, B). This may be due to the lower concentration of available fertilizer (NH_4Cl) in the latter. The decrease in the pH of the soil caused by NH_4Cl increased the availability of Zn, which is not readily available in high pH soils (Singh and Raj, 1994; Tang et al., 1995; Jeong and Lee, 1996; Barsoom, 1996). Zn plays a role in the phosphorylation process and serves as a bridge between the pyrophosphate structure of ATP or ADP and the enzyme molecules. It stabilizes the ribosome particles in the configuration of protein synthesis. Carbonic anhydrase is known to be specially activated by Zn (Tisdale et al., 1985; Jones et al., 1991; Moreno et al., 1996). Zn also compensates for some effects of stress.

The trend in boron (B) uptake as influenced by NH_4Cl application differed from that of Fe, Zn and Cu. In the cotton stem, the B content decreased at T_1 (6 kg NH_4Cl ha^{-1}), while it gradually increased with a further increase in the level (Fig. 5C), showing higher concentrations than the control at 18 kg NH_4Cl ha^{-1} from both sources. In the leaves the B content increased up to T_2 (12 kg NH_4Cl ha^{-1}), while at the highest level, T_3 (18 kg NH_4Cl ha^{-1}), it decreased (Fig. 5D). Moreover, NH_4Cl of AnalaR grade induced more B uptake than commercial fertilizer. The decrease in the B content in cotton stems may be attributed to the rapid translocation of B from stem to leaves by increasing its mobility in treated plants. AnalaR grade NH_4Cl performed better by enhancing B uptake and translocating it to the leaves. The higher B content in the leaves may be due to its higher requirements in metabolic processes there. The decrease in B uptake at T_3 (18 kg NH_4Cl ha^{-1}) may be due to a decrease in mobility after reaching a maximum threshold level. The B content remained below the toxic level in all the treatments. B is an essential element because it is involved in the synthesis of one of the bases (uracil) of RNA. It is also involved in cell division, cell differentiation, maturation, respiration and plant growth. Boron has long been associated with pollen germination and growth and it improves the stability of pollen tubes. It is relatively immobile in plants and is transported primarily in the xylem (Tisdale et al., 1985; Jones et al., 1991). Immobility resulting in the unavailability of B is more severe at higher soil pH. The decrease in this soil factor by NH_4Cl may have been the major reason for the increase in B uptake (Moreno et al., 1996).

From the results of the present study it can be concluded that the application of NH_4Cl at 18 kg ha^{-1} is beneficial in reducing the soil pH and increasing plant growth, cotton yield and nutrient availability. However, further investigations will be required to study the influence of higher rates of NH_4Cl on soil pH, EC, plant growth and nutrient uptake and the possible adverse effects of NH_4Cl.

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EFFECT OF SEASONAL VARIATION ON THE COPPER STATUS IN A SOIL–PLANT–ANIMAL SYSTEM

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A study was conducted on the sheep farm of the Livestock Experimental Station, located in the southwestern Punjab, Pakistan, to determine the copper nutrition status of different classes of grazing sheep during two different seasons. A complete free-choice supplement (feed) was available to all animals throughout the year. The purpose of this research was to investigate, as a function of the seasons, the transfer of Cu from soil, and dietary factors to sheep grazing in this semiarid region, in order to evaluate if the Cu requirement of grazing livestock was met or if a deficiency occurred. The final goal was to maximize the production of the animals by adopting, if necessary, adequate, balanced Cu supplementation. Soil, forage, feed and water samples, and animal samples (plasma, milk, faeces and urine from lactating ewes, plasma, faeces and urine from non-lactating ewes and plasma and faeces from male animals) were taken eight times during the year (four times in each season). Soil copper was affected by the seasonal changes and sampling intervals and was significantly higher than plant needs during both seasons, while the forage copper level did not show significant seasonal fluctuations, but was only affected by the sampling intervals. The soil and forage Cu was sufficient for the requirements of the plants and the animals grazing there on during both seasons. The copper contents of the feed and water showed no seasonal or sampling interval fluctuations. The plasma Cu was affected by seasonal variations in non-lactating ewes and in rams and by sampling intervals in the lactating ewes. Faecal and urine Cu was not affected by seasonal or sampling intervals except in non-lactating ewes, where the sampling interval had a pronounced effect on faecal Cu, while milk Cu in lactating ewes was affected by seasonal changes only. In all classes of sheep plasma Cu was higher during the winter than during the summer and remained in the normal range for ruminants during both seasons. It is concluded that a mixture with high bioavailability, containing Cu, should be continuously provided to grazing sheep in this semi-arid region in order to maintain the normal level of Cu and maximize the production potential of ruminants.

Keywords: seasonal variation, soil– plant– animal system, sheep, Pakistan

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Introduction

Animal nutritionists and livestock producers have recognized that variation in the nutrient profiles of feedstuffs is a common occurrence. However, few producers realize that the normal variation in energy, protein or macrominerals is relatively small compared to what has recently been reported for trace minerals (McDowell, 2003). The identification of factors contributing to this variation may be helpful to individual producers and nutritionists in preventing trace mineral deficiencies (Underwood and Suttle, 1999). Copper was the first to be recognized as an essential nutrient for animals. Today, copper deficiency is known to cause anaemia, diarrhoea, bone disorders, neonatal ataxia, changes in hair and wool pigmentation, infertility, cardiovascular disorders, impaired glucose and lipid metabolism and a depressed immune system (Davis and Mertz, 1987). Copper is a key component of many enzyme systems which, when impaired, can directly or indirectly cause many of the symptoms of copper deficiency.

Because many of the copper deficiency symptoms are general in nature, a clear diagnostic tool that accurately reflects the copper status of the animal is needed. Although serum and plasma copper concentrations are often measured, blood levels may not show the deficiency until severe symptoms develop (Hemken et al., 1993). Liver copper concentration is probably the most sensitive indicator of changes in copper status and its determination is recommended when liver biopsies can be obtained. Ceruloplasmin concentrations and superoxide dismutase activity in the blood or red blood cells can be useful indicators of copper status (McDowell, 2003).

Dietary copper requirements vary greatly among species. The recommended levels for one species may cause toxicity in another. For example, 10 ppm is the recommended level for dairy cattle, but under certain conditions 10 ppm can cause toxicity in sheep (Church and Pond, 1988). By comparison, growing pigs are often fed 100 to 250 ppm of copper in the diet to improve growth. According to the National Research Council, poultry require approximately 8 ppm copper (NRC, 1984).

Sheep are unique in that they accumulate copper in the liver more readily than other farm animals. Over a period of time, 1,000–3,000 ppm copper on a dry basis may be achieved. Usually there are no clinical signs until there is a sudden release of copper into the blood. Plasma copper levels then increase 10- to 20-fold. These elevated blood copper concentrations (500–2000 mg/dl) usually precede clinical signs by 24 to 48 hours (Underwood and Suttle, 1999). The most common symptoms are anorexia, excessive thirst and depression. Most sheep will die within 2 to 4 days after blood concentrations skyrocket.

Because of the variation in recommended copper concentrations, it is difficult to develop trace mineralized salts with a single copper level for all species. One alternative is to have a low-copper product for sheep and a high-copper product for other species. This would insure that all species would receive an appropriate amount of copper without the risk of copper toxicity in sheep. Swine producers feeding copper as a growth promotant will continue to supplement copper in addition to that in the trace mineralized salt (NRC, 1984; Peterson et al., 2000).

The long-term, heavy demands of high-yielding crops on the soil nutrient supply (primary minerals and organic matter) may show up as shortages of micronutrients (iron, manganese, zinc, copper, boron, nickel, molybdenum, etc.). Many soils are naturally low in available levels of one or more of these elements, but heavy crop demands over time may increase the severity of the deficiency, and may begin to exhaust the soil's ability to supply sufficient quantities of other micronutrients. Such deficiencies, if mild, often do not show visible symptoms in the plants. A slight yield decline may or may not be noticed. Soil and plant tissue testing are needed to verify these mild deficiencies (Singer and Ewing, 2000). Many farmers do not perform these micronutrient tests on plants and soils until the deficiencies become severe enough to be noticed (Rhue and Kidder, 1983).

Knowing the copper concentration in a diet without knowing the source of supplemental copper is of little nutritional value. The absorption of copper may vary from zero to as high as 75% (Linder, 1991) depending on a number of factors. Copper availability in most feedstuffs fed to farm animals is between 1% and 15% (Hemken et al., 1993). Grains are lower in copper than are forages. Most forages will contain copper at levels equal to or above the NRC requirement for ruminants. However, as plants mature and the phytate and lignin content increases, the bioavailability of the copper decreases rapidly. Blood copper levels were found to be a poor indicator of copper status (McDowell and Valle, 2000).

The formation of totally unavailable thiomolybdates due to the complexing of molybdenum, copper and sulphur is the reason that copper status is easily affected by molybdenum and sulphur levels. Thiomolybdate is formed in the rumen when sulphate is converted to sulphide, which is a key intermediate in forming thiomolybdate. (Sulphates are stable in the monogastric stomach, and so the problem does not occur in monogastric animals.) High sulphur in combination with normal or low molybdenum concentrations may reduce copper bioavailability by the formation in the rumen of copper sulphide, which is also poorly absorbed. If high sulphur is a problem, adding copper carbonate may be recommended over copper sulphate to avoid adding more sulphur to the diet (McDowell, 1997; 1999; 2003).

The stress associated with foetal development and calving may also increase the copper requirement. Recent research showed that dairy cows had significantly lower liver copper stores at calving than at later stages of lactation (Waterman et al., 1991). Hemken et al. (1993) reported that at least 15 ppm copper in the diet is required to replenish the mothers' liver stores, because the foetal liver takes up copper more rapidly than the mother. These data suggest that the10 ppm copper requirement prescribed by NRC may not be adequate during late gestation, when there is rapid foetal development (Underwood and Suttle, 1999).

Keeping in view the importance of Cu in animals, the present study was conducted to locate Cu deficiency or excess for grazing livestock by the use of pasture and animal samples. The intention was to document the success of the general feeding strategy for free-grazing sheep rather than to measure the exact daily Cu intake per animal. The final goal is to meet the Cu needs of grazing livestock in order to maximize the production of animal products by ensuring adequate, balanced Cu supplementation.

Materials and methods

Soil, forage, feed, water and animal samples were taken from the Livestock Experimental Station located in southern Punjab, owned by the Government of Punjab, Pakistan. These collections were made fortnightly eight times during the year (four times in summer and four in winter). Composite soil and forage samples were collected from three sites on the pasture. Five sub-samples of soil and forage were taken from the beginning, middle and end of the pasture.

Sample collection

Each composite soil sample was derived from five sub-samples, taken at a depth of 20 cm as described by Sanchez (1976). Each composite forage sample also consisted of five sub-samples of the same predominant forage species most frequently grazed by sheep on the farm. Forage samples were collected after careful observation of the sheep grazing pattern. The forage samples were clipped at a height of 3–6 cm, from the ground to simulate the grazing behaviour of the animals. Individual forage samples were collected at the same spots from where the soil samples were collected. Representative samples of the forages were then placed in polyethylene bags in the laboratory, where they were given a rapid wash with tap water followed by glass-distilled water to remove any soil which was present. The soil and forage samples were then placed in clean cloth bags for air drying.

For sampling purposes the animals were divided into 3 classes, lactating and non-lactating ewes and male animals, respectively, with 10 animals per class. Blood plasma, milk, faeces and urine samples were taken from lactating ewes, plasma, faeces and urine from non-lactating ewes and plasma and faeces from male sheep, concurrently with the soil and forage samplings.

Blood samples were anaerobically collected by jugular vein puncture with a syringe and needle, then drawn by vacuum into evacuated tubes containing lithium heparine as an anticoagulant. The plasma was separated by centrifugation, harvested in polyethylene tubes and frozen at –20°C for subsequent analysis for copper. Faecal samples were collected from the rectum of the animals manually and urine samples via manual stimulation of the vulva of female animals, after which 10 ml aliquots were transferred to polyethylene tubes, acidified with 0.3 ml concentrated HCl, and frozen for subsequent analysis (Tucker et al., 1991). The faecal samples were kept in open bags and allowed to dry in the sun to constant atmospheric moisture (<30%). Milk samples were collected in 125 ml nalgene bottles using the first drawn milk. All lactating animals were sampled shortly after the administration of a 1 ml oxytocin injection to stimulate milk let-down. Milk samples were taken in plastic vials and stored frozen until analysis (Fick et al., 1979).

Samples of the feed consumed by the animals were collected in five replicates for copper assay at each sampling period and air-dried in cloth bags. Water samples were taken in borosilicate vials fortnightly during both sampling seasons simultaneously with the other samples in five replicates. The samples of forage, feed and faeces were dried in an oven at 60°C for 48 h.

Sample preparation

Air- and oven-dried soil samples were pulverized in a ceramic mortar to pass through a 2 mm sieve and were analysed for Cu concentrations using the Mehlich-1 extraction procedure (Hesse, 1972; Rhue and Kidder, 1983): 5 g of soil were added to 20 ml of 0.05 M HCl in 0.025 M H_2SO_4 and the final volume was analysed.

Water and urine samples were filtered into sterilized plastic beakers, and 1 ml aliquots were used to prepare serial dilutions for analysis. Air- and oven-dried samples of forage, feed and faeces were ground with a Wiley mill to fit through a 1 mm mesh. To prepare samples for the estimation of copper, representative dried and ground samples of about 2 g each of forage, feed and faeces were digested with nitric acid and perchloric acid (3:1) at 250°C until the solution changed to colourless and thick white fumes appeared in the flask. The contents of the flask were washed with pure water and diluted to constant volume. The supernatant obtained from centrifugation was used for analysis (Koh and Judson, 1986; AOAC, 1990; Neatheary et al., 1990). Direct dry or wet ashing of plasma and milk was not possible because of the high fat, protein and moisture, as spattering and swelling might result in loss of sample. Therefore, appropriate quantities of each plasma and milk sample were placed in a crucible after thawing. After pre-digestion with 50% HNO_3 over an electric heater until smoking ceased, to char the majority of organic matter, the samples were ashed for 6 hours at 550°C in a muffle furnace.

The residues were dissolved in 1% HCl, transferred to a volumetric flask and made up to a constant volume of 50 ml. The samples were poured into labelled plastic tubes to fit the autosampler of the atomic absorption spectrophotometer. The samples were diluted to determine individual elements (Fick et al., 1979; AOAC, 1990; Nockels et al., 1993; Mpofu et al., 1999).

All the samples were filtered through Whatman filter paper No. 42, brought to the appropriate volume with double-distilled water and stored in polyethylene tubes. The samples were analysed for Cu concentration by atomic absorption spectrophotometry (Perkin-Elmer Model 5000).

Statistical analysis

The data were analysed using a split-plot design (Steel and Torrie, 1980). Differences between means were ranked using Duncan's New Multiple Range Test (Duncan, 1955).

Soil, forage and plasma copper concentrations were compared to established critical values to determine deficiency categories. The critical soil level is the copper concentration below which normal growth and/or mineral composition of the forage may be adversely affected. For forage samples, it indicates the lowest requirement of the element or organic constituent to avoid deficiency symptoms in animals. Critical plasma levels indicate the concentration below which specific signs of deficiency may occur. The interpretation of these critical values was done with caution, taking into consideration the management, nutritional, environmental and individual factors that affect the availability, supply and utilization of copper.

Results

Pasture samples

Soil

The Cu^{2+} concentration in the soil varied ($p<0.001$) in different seasons and in different fortnightly periods (Table 1). The soil Cu^{2+} was significantly higher in summer than in winter and remained unchanged in different fortnights during winter. In contrast, during summer the soil Cu^{2+} was higher in the first fortnight than in the other three fortnights (Fig. 1a).

Table 1

Analysis of variance on the Cu^{2+} concentration in soil, forage plants, water and feed in different fortnights during the winter and summer seasons on a sheep ranch

Source of variation	Degrees of freedom	Mean squares		Degrees of freedom	Mean squares	
		Soil	Forage plants		Water	Feed
Season (S)	1	4.5***	1.04^{ns}	1	0.00016^{ns}	275.6^{ns}
Error	28	0.09	38.02	8	0.00026	85.5
Fortnight (FN)	3	0.35***	68.57**	3	0.00026^{ns}	22.4^{ns}
S × FN	3	0.13**	9.25^{ns}	3	0.00001^{ns}	66.4^{ns}
Error	84	0.03	12.35	24	0.00012	25.4

, * = Significant at the 0.01 and 0.001 levels, respectively; ns = non-significant

Forage plants

Considerable variation in forage Cu^{2+} was observed at different sampling intervals ($p<0.01$), but no seasonal effect was noted ($p>0.05$) (Table 1). Non-consistent fluctuations were observed in the forage Cu^{2+} level for different fortnights during both seasons (Fig. 1b). A higher concentration of forage Cu^{2+} was found in the first fortnight during both seasons than in the last three fortnights.

Water

Neither season nor fortnight had any significant effect ($p>0.05$) on the Cu^{2+} concentration in the water (Table 1). Generally, higher Cu^{2+} concentration was found in the water in winter than in summer (Fig. 1c). Non-consistent fluctuations in water Cu^{2+} were found in winter and summer.

Feed

The Cu^{2+} concentration in the feed was slightly lower at all sampling intervals in winter as compared to that in summer, although no significant seasonal difference was found ($p>0.05$) (Table 1). The fortnights did not differ significantly with respect to feed Cu^{2+} either (Fig. 1d). A non-consistent pattern of increase or decrease in feed Cu^{2+} was found during both seasons.

Animal samples – Lactating sheep

Plasma

From analysis of variance it is evident that the season ($p<0.001$) and fortnights ($p<0.05$) had a significant effect on the plasma Cu^{2+} (Table 2a). The Cu^{2+} level was markedly higher in winter than in summer, but there were non-consistent fluctuations for the different fortnights in winter (Fig. 2a). In contrast, a consistent increase in plasma Cu^{2+} was observed during the summer.

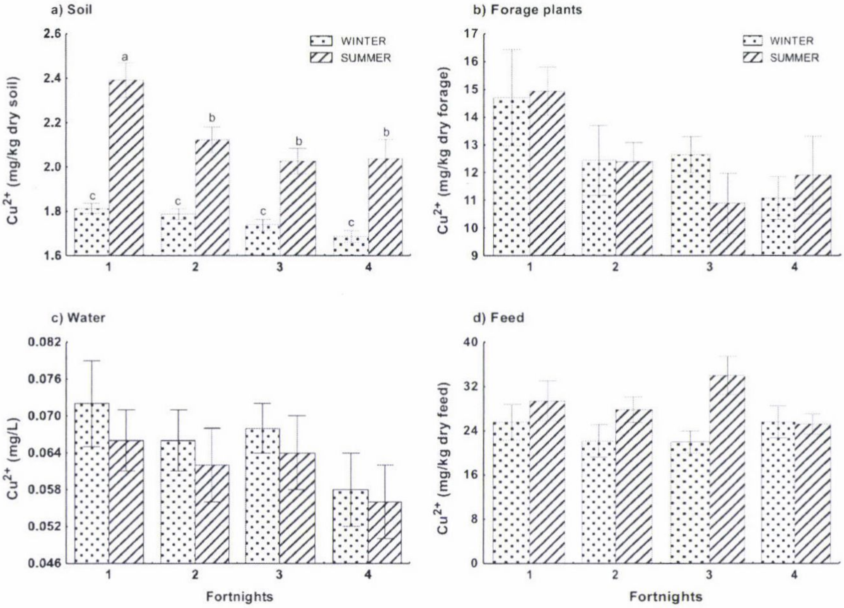

Fig. 1. Cu2$^+$ concentration in soil (a), forage plants (b), water (c) and feed (d) in different fortnights during the winter and summer seasons (sheep farm). Means with the same letters did not differ significantly at $p \leq 0.05$

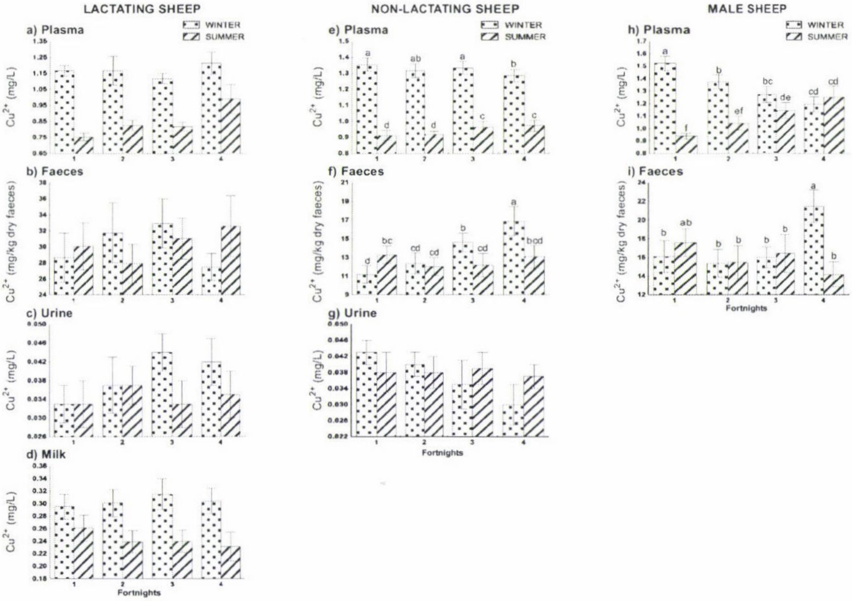

Fig. 2. Cu2$^+$ concentration in different sample types of lactating, non-lactating and male sheep in different fortnights during the winter and summer seasons. Means with the same letters did not differ significantly at $p \leq 0.05$

Faeces

There was no significant effect of seasons or fortnights ($p>0.05$) on faecal Cu^{2+} (Table 2a). There was a substantial increase in faecal Cu^{2+} with the time of sampling in winter up to the third fortnight, but there was a sharp reduction in the fourth fortnight. In contrast, the Cu^{2+} level increased gradually with time except for the second fortnight in summer, where a marked reduction in faecal Cu^{2+} was found (Fig. 2b).

Urine

The Cu^{2+} concentration in urine was not affected by either seasons or fortnights ($p>0.05$) (Table 2a). In winter, the urine Cu^{2+} increased from the first to the third fortnights, but in the fourth fortnight the urine Cu^{2+} was almost equal to that in the third fortnight. In contrast, in summer the pattern of increase or decrease in urine Cu^{2+} level in different fortnights was non-consistent (Fig. 2c).

Milk

There was a significant seasonal effect on the milk Cu^{2+} level ($p<0.05$), but the fortnights had no effect ($P>0.05$) (Table 2a). The excretion of Cu^{2+} via milk was generally higher in winter than in summer (Fig. 2d). A uniform level of Cu^{2+} in milk was observed in all fortnights during the winter, while in summer a consistent decrease was observed with time.

Non-lactating sheep

Plasma

Significant seasonal ($p<0.001$) and non-significant ($p>0.05$) fortnightly effects were observed on the plasma Cu^{2+} concentration (Table 2b). The Cu^{2+} level was markedly higher in winter than in summer. During the winter, there was no significant change with time up to fortnight 3, but the Cu^{2+} level decreased in fortnight 4, whereas in summer the Cu^{2+} level was uniform during the first two fortnights, rising to a higher uniform level in the last two fortnights (Fig. 2e).

Faeces

The season had no effect ($p>0.05$) on the Cu^{2+} concentration, whereas the sampling period influenced the Cu^{2+} concentration significantly ($p<0.001$) (Table 2b). There was a sharp increase in Cu^{2+} concentration in winter with the time of sampling, but during the summer no consistent pattern of increase or decrease in faecal Cu^{2+} was found (Fig. 2f).

Urine

No significant seasonal or sampling period effects ($p>0.05$) on urine Cu^{2+} concentration were found (Table 2b). In winter, the Cu^{2+} level decreased with time, while in summer there was no change in the urine Cu^{2+} in different fortnights (Fig. 2g).

Table 2a

Analysis of variance on the Cu^{2+} concentration in the blood plasma, faeces, urine and milk of lactating sheep in different fortnights during the winter and summer seasons

Source of variation	Degrees of freedom	Mean squares			
		Plasma	Faeces	Urine	Milk
Season (S)	1	2.04***	0.099^{ns}	0.00041^{ns}	0.074*
Error	18	0.04	200.9	0.00024	0.02
Fortnight (FN)	3	0.09*	26.11^{ns}	0.00014^{ns}	0.001^{ns}
S × FN	3	0.03^{ns}	76.25^{ns}	0.00015^{ns}	0.002^{ns}
Error	54	0.03	51.20	0.00023	0.001

*, **, *** = Significant at the 0.05, 0.01 and 0.001 levels, respectively; ns = non-significant

Male sheep

Plasma

The Cu^{2+} availability in the blood plasma was affected ($p<0.01$) by the season, but the sampling interval had no significant effects ($p>0.05$) (Table 2b). There tended to be a gradual decrease in plasma Cu^{2+} over time in winter and an increase in summer (Fig. 2h). The bioavailability of Cu^{2+} in the plasma was significantly higher in winter as compared to summer.

Faeces

No significant effect of seasons or fortnights was observed ($p>0.05$) on the faecal Cu^{2+} concentration (Table 2b). There were great fluctuations in Cu^{2+} concentration in different fortnights during the summer and winter seasons except for the fourth fortnight of winter, when the faecal Cu^{2+} was the highest of all the fortnights in both seasons (Fig. 2i).

Discussion

This investigation was performed in a semi-arid region of Punjab State in Pakistan, the climate of which undoubtedly influences the mineral composition of soils and forage crops, as well as animal metabolism. Therefore, the results obtained are only valid for grazing animals living in such regions under similar tropical or subtropical conditions.

The soil Cu^{2+} levels during the winter and summer seasons were above the critical level for the normal growth of plants (Rhue and Kidder, 1983). Similar levels of soil Cu^{2+} in the winter and summer seasons were earlier reported in Colombia (Pastrana et al., 1991), Guatemala (Tejada et al., 1987) and Nicaragua (Velasquez-Pereira et al., 1997). Horowitz and Dantas (1973) suggested that soils with less than 0.6 mg/kg of extractable Cu^{2+} were deficient for pasture and crops. On this basis, the mean values of soil Cu^{2+} recorded in the present study were not deficient.

Table 2b

Analysis of variance on the Cu^{2+} concentration in the blood plasma, faeces and urine of non-lactating ewes and in the plasma and faeces of male sheep in different fortnights during the winter and summer seasons

Source of variation	Degrees of freedom	Mean squares				
		Non-lactating sheep			Male sheep	
		Plasma	Faeces	Urine	Plasma	Faeces
Season (S)	1	2.89***	25.31^{ns}	0.00020^{ns}	1.22**	34.45^{ns}
Error	18	0.05	40.65	0.000312	0.102	38.73
Fortnight (FN)	3	0.003^{ns}	34.28***	0.000183^{ns}	0.003^{ns}	28.59^{ns}
S × FN	3	0.014***	33.18***	0.000150^{ns}	0.38***	81.44*
Error	54	0.002	3.91	0.000153	0.02	21.40

*, **, *** = Significant at the 0.05, 0.01 and 0.001 levels, respectively; ns = non-significant

The forage and feed Cu^{2+} concentrations were found to be sufficiently high to meet the demands of the animals during both seasons. In addition, the water Cu^{2+} contents during both seasons seemed to be just as important as the forage Cu^{2+} concentrations for ruminant needs. The forage Cu^{2+} had no relationship with soil Cu^{2+} levels during either season. The forage Cu^{2+} values found in this study were not higher than those reported previously in north Florida (Tiffany et al., 2001), Venezuela (Rojas et al., 1993) and central Florida (Espinoza et al., 1991).

These values were similar to those reported for Indonesia (Prabowo et al., 1991) and lower than those reported by Tejada et al. (1985, 1987) in Guatemala. The low forage Cu^{2+} observed in this study may have been due to its interaction with other elements in the soil. McDowell et al. (1993) reported that Cu^{2+} interacts strongly with trace minerals and macro-minerals. Fe^{2+} and Ca^{2+} are among the elements that could have had an effect on the absorption of Cu^{2+}', because the concentrations of these elements were very high. Ca present in the carbonate form precipitates Cu^{2+}, making it unavailable for the plants. In addition, the content of this element is often inversely related to increasing plant maturity, possibly one of the causes of low levels of Cu^{2+} in the forage (McDowell et al., 1983).

The Cu^{2+} sources were not significantly different during the two seasons, but during summer a slightly higher concentration was found than in winter. The plasma Cu^{2+} concentrations of all classes of sheep were significantly higher in winter than in summer, showing seasonal as well as physiological effects. Higher plasma Cu^{2+} was found in male sheep than in lactating and non-lactating ewes during both seasons. The low plasma Cu^{2+} level in lactating sheep may have been due to its secretion in milk and faeces, because the faecal Cu^{2+} excretion in this group was highest of all the classes of sheep. Almost the same amount of urine Cu^{2+} excretion was found in lactating and non-lactating sheep. In the winter season, feed and forage Cu^{2+} contributed to enhancing the plasma

Cu^{2+} levels, but in summer the feed Cu^{2+} level, although very high, was ineffective in elevating plasma Cu^{2+} levels in all classes of sheep.

The low plasma Cu^{2+} levels were not due to the Cu^{2+} status in the diet, since the forage and feed collectively had higher Cu^{2+} content in summer than in winter, demonstrating that the source Cu^{2+} level was unable to raise the plasma Cu^{2+}. Nevertheless, plasma Cu^{2+} concentrations were above the critical level in all classes of sheep during both seasons (McDowell, 1985).

High levels of plasma Cu^{2+} in winter were previously reported in sheep in Colombia (Pastrana et al., 1991) and in cattle in Nicaragua (Velasquez-Pereira et al., 1997). According to Suttle (1986) and Mills (1987) the plasma Cu^{2+} is of limited value in diagnosing Cu^{2+} status, because of certain diseases responsible for altering these levels. In this study there were small seasonal differences in the concentration of dietary Cu^{2+}, which seemed to have no effect on the plasma Cu^{2+} of the sheep. This was in agreement with the observation of Underwood (1981) that plasma/serum Cu^{2+} does not reflect dietary Cu^{2+}, and the results obtained in the present study did not confirm the findings of Rowlands (1980), who suggested that plasma Cu^{2+} is affected by dietary Cu^{2+} intake.

Plasma Cu^{2+} levels in male animals were slightly higher during both seasons than in other groups of animals. Goodrich et al. (1972) reported that the extent of Cu^{2+} absorption may be influenced by age, some hormones, pregnancy and certain diseases. In addition, various nutrient interrelationships have been found to affect the absorption of Cu^{2+}. Cu^{2+} deficiency is widespread in grazing ruminants throughout the world (McDowell, 1985). Most deficiencies are conditioned by the presence of dietary factors, which interfere with the utilization of Cu^{2+} by the animals. The low level of plasma Cu^{2+} found in this study during the summer may have been due to its rapid tissue distribution and to the inhibitory effect of Fe^{2+}, and to the active process of its excretion through faeces and urine in summer.

Conclusions

This study indicated that seasonal fluctuations were only found in soil, milk and plasma Cu^{2+} concentrations in all classes of sheep. Higher plasma levels were found in winter than in summer. Although an adequate level of plasma Cu^{2+} was found, it was bordering on deficient levels. Thus, supplementation is needed with a mixture containing Cu^{2+}.

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INFLUENCE OF OMITTING IRRIGATION AND NITROGEN LEVELS ON GROWTH, YIELD AND WATER USE EFFICIENCY OF CORIANDER (*Coriandrum sativum* L.)

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A field experiment conducted on the sandy loam soil in New Delhi during the winter season of 2003–2004 indicated that the application of three irrigations at the branching, flowering and seed formation stages gave the maximum values of growth and yield attributes (plant height and branches plant^{-1}, umbels plant^{-1}, umbellets umbel^{-1} and seeds umbel^{-1}) with the highest seed and stover yields, but was at par with omitting one irrigation at the seed formation stage. The data on the growth and yield indicated that, compared to the other stages, omitting irrigation at the flowering strage resulted in the greatest reduction. The growth and yield attributes and the seed and stover yield of coriander significantly responded to fertilization up to 80 kg N ha^{-1}. The crop evapotranspiration was the highest with the application of three irrigations, but the water use efficiency remained the highest when irrigation was omitted at the seed formation stage. Nitrogen fertilization up to 120 kg and 80 kg N ha^{-1} increased the water use and water use efficiency, respectively.

Key words: coriander (*Coriandrum sativum*), moisture regimes, nitrogen, yield, water use efficiency

Introduction

Coriander (*Coriandrum sativum* L.) is an annual herb, originating in the Mediterranean region. It has an erect branching stem growing to a height of about 30 to 70 cm with compound leaves. It is mainly grown for seeds, which have a fragrant odour and pleasant aromatic taste and contain 0.1 to 1.0% essential oil. Coriander seed and the oil obtained from them are used in the medicinal, food and perfume industries. Coriander is one of the principle ingredients of curry and of other spice mixtures. In some countries young leaves of coriander are also used to flavour salad and soups.

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Coriander is widely adapted to a variety of climate and soil types in India, Morocco, Russia, Hungary, Poland, Romania, Guatemala, Mexico and the USA. In India, coriander occupies 0.42 million hectares with an annual production of 0.25 million tonnes of seed and is mainly grown during the winter season on the northwestern plains. The productivity of coriander seed is 595 kg ha^{-1} in India, which is very low. One of the main reasons for the low productivity is that this crop is grown in areas characterized by light soils with medium fertility and limited availability of irrigation water. Among the different inputs, water and nitrogen play a vital role in maximizing the yield potential of the coriander crop. Coriander requires about 400 mm of water (Luayza et al., 1996) and responds well to irrigation application at various growth stages (Sharma and Israel, 1991; Singh et al., 2002). Buntain and Chung (1994) opined that irrigation application at the flowering stage is most important in fennel. Similarly, Barreyro et al. (1993), Garg et al. (2004) and Oliveira et al. (2003) found that nitrogen had a role in improving the productivity of coriander. Further, Luayza et al. (1996) advocated that for higher production coriander requires fertile soils. Little information is available on the response of coriander to omitting irrigation at varying nitrogen levels. It is thus imperative to discover which growth stages of coriander can tolerate the omission of irrigation without a drastic reduction in yield and to calculate the nitrogen requirements of coriander. Hence, the present study was undertaken to demonstrate the effect of omitting irrigation in coriander (*Coriandrum sativum* L.) at different stages and at varying nitrogen levels.

Materials and methods

The experiment was conducted during the winter season of 2003–2004 on sandy loam soil in New Delhi. The soil was low in available nitrogen (178 kg N ha^{-1}), medium in available phosphorus (15.6 kg P ha^{-1}) and high in available potassium (234 kg K ha^{-1}) with pH 8.1. The experiment was laid out in a split-plot design and replicated thrice. The treatments comprised five moisture regimes (T1: no irrigation after sowing; T2: three irrigations at the branching, flowering and seed formation stages; T3: omitting one irrigation at branching; T4: omitting one irrigation at flowering; T5: omitting one irrigation at the seed formation stage) in the main plots and four levels of nitrogen (0, 40, 80 and 120 kg N ha^{-1}) in the sub-plots. Coriander cultivar RCr 1 was sown on 27th November 2003 after applying pre-sowing irrigation and harvested on 8th April 2004. The optimum plant population was maintained at row-to-row and plant-to-plant spacings of 30 and 15 cm, respectively. All plots were given 17.5 kg P and 24.9 kg K ha^{-1} and half the prescribed amount of nitrogen was applied as basal fertilizer, while the remaining nitrogen was top-dressed at the four-week crop stage. The observations on growth and yield parameters were made at the harvest stage from five plants, sampled from the second row of each plot. To estimate the evapotranspiration, soil samples up to 120 cm depth were taken at the time of sowing and harvesting, and before and after each irrigation. The value of evapotranspiration was determined by the method of Dastane (1972) from the soil moisture data.

Results and discussion

Growth parameters

The plant height and number of branches plant^{-1} of coriander differed significantly in the various moisture regimes (Table 1). The tallest plants with the maximum number of branches plant^{-1} were obtained with the application of three irrigations at the branching, flowering and seed formation stages. Omitting irrigation at the branching stage markedly reduced both plant height and number of branches plant^{-1}, which may be due to the lower availability of water and poor nutrient uptake at that stage. Tomar et al. (1994) also reported similar findings in coriander. Omitting irrigation at the seed formation stage, however, did not reduce the plant height or the number of branches plant^{-1}. Each successive increase in the nitrogen level from 0 to 80 kg N ha^{-1} enhanced the plant height, while the number of branches plant^{-1} increased up to 120 kg N ha^{-1} (Table 1). Cells developed at increasing nitrogen levels tended to be large (Black, 1967) with higher meristematic activities, which consequently benefited the growth. Barreyro et al. (1993) and Rahman et al. (1990) reported a similar trend.

Yield attributes

The yield attributes (umbels plant^{-1}, umbellets umbel^{-1} and seeds umbellet^{-1}) were favourably influenced by the application of three irrigations at the branching, flowering and seed formation stages (Table 1). However, omitting irrigation at the flowering stage reduced the umbels plant^{-1}, umbellets umbel^{-1} and seed umbellet^{-1}, indicating that omitting irrigation at the flowering stage is the most harmful. Similarly, omitting irrigation at the branching stage also caused a decline in umbels plant^{-1} and seeds umbellet^{-1}, while omitting irrigation at the seed formation stage did not affect umbels plant^{-1} or umbellets umbel^{-1}. The higher physiological activity and better growth of crop plants with irrigation application at the different stages, particularly at flowering, might have enhanced the supply of photosynthates from source to sink, consequently increasing the production of yield attributes with more frequent irrigation. Other findings on the yield attributes of coriander (Tomar et al., 1994) and fennel (Buntain and Chung, 1994) confirmed these results. The 1000-seed weight remained unaffected by variations in the moisture regime. Each successive increase in the nitrogen level from 0 to 80 kg ha^{-1} led to a marked increase in the umbels plant^{-1}, umbellets umbel^{-1}, seeds umbellet^{-1} and 1000-seed weight, but a further increase in the nitrogen level from 80 to 120 kg ha^{-1} did not result in a significant improvement in these yield attributes (Table 1). Vigorous vegetative growth and a better supply of photosynthates to the sink at higher nitrogen levels might have resulted in these increased yield attributes. Sharma and Israel (1991) also found higher values of yield attributes with increasing nitrogen levels.

Table 1

Growth and yield attributes of coriander as influenced by moisture regimes and nitrogen levels

Treatments	Plant height (cm)	Branches plant^{-1}	Umbels plant^{-1}	Umbellets umbel^{-1}	Seeds umbellet^{-1}	1000-seed weight (g)
Moisture regimes						
T1	55.0	5.9	14.5	3.5	11.0	10.2
T2	68.1	7.9	17.0	4.8	13.3	11.8
T3	65.2	6.2	15.7	4.6	12.1	11.0
T4	66.0	7.3	15.1	4.5	11.8	10.5
T5	67.3	7.5	16.3	4.7	12.4	11.2
CD (5 %)	2.7	0.6	1.1	0.3	0.9	NS
Nitrogen (kg/ha)						
0	56.6	6.0	14.5	3.7	11.2	9.8
40	63.9	6.8	15.6	4.5	12.0	10.8
80	67.8	7.3	16.3	4.7	12.6	11.5
120	69.0	7.7	16.5	4.8	12.7	11.7
CD (5%)	1.3	0.4	0.6	0.2	0.4	0.4

T1: No irrigation after sowing; T2: Three irrigations at the branching, flowering and seed formation; T3: Omitting one irrigation at branching; T4: Omitting one irrigation at flowering; T5: Omitting one irrigation at seed formation; NS = non-significant

Yield

In general, compared to three irrigations, omitting irrigation at the branching, flowering or seed formation stages and applying no post-sowing irrigation reduced the seed yield by 16.8, 18.9, 12.2 and 36.2% and the stover yield by 17.1, 17.5, 8.0 and 39.2%, respectively (Table 2). The application of three irrigations at the branching, flowering and seed formation stages produced significantly greater seed and stover yields than all the other moisture regimes except for omitting irrigation at the seed formation stage. The findings of Singh et al. (2002) and Tomar et al. (1994) confirmed these results. No significant variations in seed or stover yields were observed between the treatments that omitted irrigation at branching or flowering or seed formation, but the greatest reduction in yield was found when irrigation was omitted at flowering. Buntain and Chung (1994) also found flowering to be the most critical stage for irrigation in fennel. Nitrogen application at 40, 80 and 120 kg N ha^{-1} produced 29.6, 44.8 and 46.4% higher seed yield and 49.7, 70.7 and 78.2% higher stover yield, respectively, compared to no nitrogen application (Table 2). However, significant increases in seed and stover yield were only found up to 80 kg N ha^{-1} levels. Barreyro et al. (1993), Garg et al. (2004) and Oliveira et al. (2003) also reported the positive response of coriander to nitrogen application.

Water use studies

The maximum (382.8 mm) and minimum (275.4 mm) values of evapotranspiration by coriander were recorded for three irrigations and for no post–sowing irrigation, respectively (Table 2). When irrigation was omitted either at branching or flowering or seed formation evapotranspiration remained more or less similar, but lower than the three irrigations. The water use efficiency was the highest (5.23 kg ha^{-1} mm^{-1}) when irrigation was omitted at the seed formation stage, followed by the application of three irrigations (5.12 kg ha^{-1} mm^{-1}). Similarly high water use efficiency was reported after the application of two irrigations in coriander by Tomar et al. (1994). The lowest water use efficiency (4.54 kg ha^{-1} mm^{-1}) was recorded in the no post-sowing irrigation treatment. With regard to nitrogen application, evapotranspiration increased with each increase in nitrogen level from 0 to 120 kg ha^{-1} (Table 2). However, the water use efficiency only improved up to 80 kg N ha^{-1}. The higher water use efficiency at higher nitrogen levels can be attributed to the fact that there was a relatively greater increase in seed yield than in evapotranspiration up to 80 kg N ha^{-1}.

On the basis of these findings it can be concluded that irrigation is important for coriander at all three stages, but omitting irrigation at the seed formation stage led to no significant reduction in yield. Irrigation should not be omitted at the flowering stage, hovewer. A nitrogen level of 80 kg ha^{-1} was found to be sufficient for coriander.

Table 2

Yield and water use by coriander as influenced by moisture regimes and nitrogen levels

Treatments	Seed yield (q ha^{-1})	Stover yield (q ha^{-1})	Evapotranspiration (mm)	WUE
Moisture regimes				
T1	12.5	16.0	275.3	4.54
T2	19.6	26.3	382.8	5.12
T3	16.3	21.8	335.6	4.86
T4	15.9	21.7	330.4	4.81
T5	17.2	24.2	328.6	5.23
CD 5%	2.9	3.2	–	–
Nitrogen (kg/ha)				
0	12.5	14.7	273.9	4.56
40	16.2	22.0	330.1	4.91
80	18.1	25.1	352.2	5.13
120	18.3	26.2	366.0	5.00
CD 5%	1.3	1.4	–	–

For moisture regimes see Table 1; WUE: Water use efficiency (kg ha^{-1} mm^{-1})

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RELATIONSHIPS BETWEEN CULTIVATION TECHNIQUES, VEGETATION, PEDOLOGY AND EROSION ON EXTENSIVELY CULTIVATED AND ABANDONED AGRICULTURAL AREAS IN THE PUTNOK HILLS

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Pedological and coenological investigations were made around the villages of Alsószuha and Gömörszőlős, in the Putnok Hills microregion, which forms part of the Northern Hungarian Mountains. These were complemented with laboratory nutrient analyses, giving the opportunity to compare the pedological relationships and erosion of natural and ploughed areas. Arable lands can often be found on steep slopes. The brown forest soil types characteristic of these areas are less sensitive to erosion, but they suffer significant damage when cash crops are hoed regularly even on steep slopes.

Coenological data indicative of the previous farming system are presented for three plots near Alsószuha. Regular mowing resulted in a large number of plant species even 10 years after cereal production was abandoned. The lack of regular mowing on a plot with a similar farming history, however, resulted in the dominance of aggressive weeds. The species on the third plot showed that the 42 years that had elapsed since cereal production was abandoned, followed by grazing until 1990, ensured enough time for revegetation and the generation of a secondary grassland (slope steppe) in a close-to-natural state. Invasive weeds were absent from all the observed plots.

Key words: pedology, coenology, erosion, land abandonment, Putnok Hills

Introduction

The studied area, the Putnok Hills microregion, is part of the Northern Hungarian Mountains mesoregion, ranging from the Sajó Valley to the southern border of the main part of the Aggtelek National Park (Marosi and Somogyi, 1990).

Traditional land use methods were observed from the point of view of their influence on habitats in a close-to-natural state. These areas are extremely important for nature conservation because valuable plant taxa can only be preserved for future generations by sustaining the management patterns used for hundreds of years. The plant communities and protected species in the studied area were described for various landscape management methods by Malatinszky (2004).

The natural conditions of the area are suited to forestry, pasture management and crop cultivation. Ancient agricultural activities carried out in diverse habitats resulted in specially structured landscape mosaics. Besides biological and landscape diversity, an adequate cultivation structure is also important for the preservation of soil fertility and to avoid erosion (Centeri, 2002a). Soil is one of the most important components of the landscape. Its preservation is a priority, because it is a non-renewable resource compared with the scale of the human lifetime. Eroded soil material may carry humus and important fertilizers. As much as 9 kg $ha^{-1}year^{-1}$ N, 5.5 kg $ha^{-1}year^{-1}$ P and 6.6 kg $ha^{-1}year^{-1}$ K may be lost due to erosion from arable lands (Centeri, 2002b). Detailed soil data for the Alsószuha research area were reported by Centeri and Császár (2005).

The preservation of the environment and of natural habitats is vital not only because of their effects on the human environment and due to the cost of recultivation after mining, floods and landslides or after preventable damage, but because nature and the natural environment are also valuable in themselves. However, it is difficult to measure the value of the landscape and express it in monetary terms (Csorba, 2003). The investigation of changes in landscape patterns is extremely important (Barczi et al., 1996/97).

Materials and methods

The investigated area is situated in the Putnok Hills microregion, near the village of Alsószuha, on the eastern slope of Lengyel-oldal hill, and on the Cuda area near Gömörszőlős.

Laboratory soil experiments

The amounts of AL-P_2O_5, AL-K_2O, $CaCO_3$ and soil organic matter and the pH (KCl and H_2O) were measured using standard procedures in the Department of Soil Science and Agricultural Chemistry of Szent István University.

Field soil experiments

Slopes with different vegetation cover were chosen for the investigations. In the Alsószuha area three different vegetation covers were examined on the same side of a hill. The slope angle was 12–17% on the lower slope and 17–25% on the upper slope (Fig. 1). In Gömörszőlős (Fig. 2) the upper slope had an angle of 12–17% and the lower slope 5–12%.

The field studies included sampling with a Pürckhauer soil core sampler (Finnern, 1994) and full soil profile descriptions (Stefanovits, 1992). Core sampling allowed numerous samples to be taken for the analysis of the depth of the layers, pH, colour, soil texture, carbonate content and soil type, while samples were taken from the profiles in order to analyse basic parameters.

Botanical investigations

Species names follow the nomenclature of Simon (2000). Rare species (including weeds) were compared not only with modern literature, but also with the Herbarium Carpato-Pannonicum collection in the Hungarian Natural History Museum. Coenological investigations were made on 14 September 2005 on patches of typical vegetation neighbouring the sampled arable land, using 2×2 m quadrates, based on the method of Braun-Blanquet (1964). The cover rates of the various species are given as percentages.

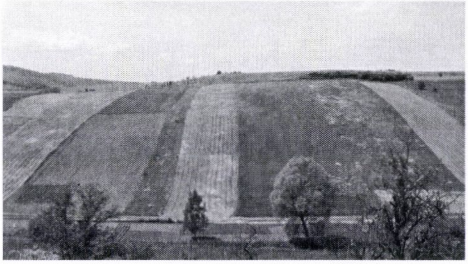

Fig. 1. Area examined near Alsószuha

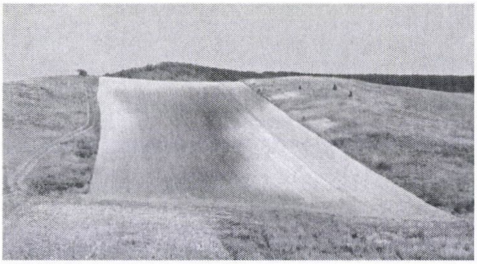

Fig. 2. Area examined near Gömörszőlős

Landscape history and management

The following sources were used for investigations on previous farming methods and the landscape history of the area: aerial photos, old military maps (on which changes in land use can be clearly traced), interviews with older inhabitants and the working plans of forest managers.

Results

An examination of the soil cover of both the Alsószuha and Gömörszőlős locations showed that they had previously been subjected to intensive land use. These areas, now used non-intensively, are the home of valuable, rare plant species and have very shallow soils, sometimes only 5–10 centimetres thick, where the loess parent rock is mixed with the humic layer. When the land has no plant cover, the effect of erosion is easily detected, because the lighter, yellowish colour of the parent material is visible on the surface.

The Gömörszőlős site was the more eroded (Fig. 2). During the spring of 2005, the plot was used for triticale production. As a result of non-intensive landscape management and the lack of pesticides, rare weed species such as *Agrostemma githago* and *Bifora radians* were found on the site. The dry slope steppes surrounding the plot were in a weedy, disturbed state, with relatively few plant species indicative of close-to-natural conditions.

The examination of the soil profile (Table 1) showed that very little of the original soil horizon remained. The top 5–15 cm layer still contained 1.57% organic matter, so the most probable scenario is that cultivation and erosion continuously mixed the upper soil layer with the lower ones, until they reached the parent material. The high $CaCO_3$ content (>20%) was also reflected by the pH (8; 8.2).

The site examined near Alsószuha (Fig. 1) was used mainly for cereal production until 1963, after which it was divided into narrow allotments. These small plots are now sown to maize, wheat, oats, alfalfa or grass, or are used as meadow (in a close-to-natural state), while many plots have been abandoned over the past ten years. The valuable plant taxa found in this area include *Euphorbia salicifolia*, *Rapistrum perenne*, *Scutellaria hastifolia* and *Dianthus deltoides*.

At this site there is still a thick soil cover above the parent material. The reason for the low erosion rate is partly due to the form of land use, as much of the weed control and soil tillage are done by hand. The other reason for the slower erosion is the shorter slope and a relatively long non-eroded plateau above the arable land that has been covered with alfalfa for the last 6 years. The laboratory data (Table 2) reflect the low-scale use of fertilizers: the AL-P_2O_5 contents of the A, B and C_1 horizons are very low. Some mixing of the soil particles (see K_A values) may have occurred, since the colour of the soil becomes lighter towards the A horizon, suggesting that the lower horizons were disturbed by ploughing or disking.

In the Gömörszőlős area, the basic soil parameters (Table 3) of an arable area were compared with those of a dry slope steppe (situated on the same slope). The differences in the laboratory data suggest that the grassed area was previously used for intensive farming. Significant differences were observed in the AL-P_2O_5 contents of the upper and lower thirds of the slope for both the arable land and the grassland.

Table 1

Laboratory data of the soil profile examined in Gömörszőlős

Soil layer	pH_{KCl}	pH_{H_2O}	$CaCO_3$ (%)	AL-P_2O_5 (mg kg^{-1})	AL-K_2O (mg kg^{-1})	SOM* (%)
A	6.87	8.03	23.9	62.23	253.83	1.57
C	6.86	8.26	23.1	37.33	147.55	0.49

*SOM = soil organic matter

Table 2

Laboratory data of the soil profile examined in Alsószuha

Soil layer	pH_{KCl}	pH_{H_2O}	$CaCO_3$ (%)	AL-P_2O_5 (mg kg^{-1})	AL-K_2O (mg kg^{-1})	SOM* (%)
A	6.07	7.37	0	24.47	127.11	1.84
B	6.54	7.81	0.5	45.26	149.88	0.58
C_1	6.65	7.95	0.6	22.35	178.62	0.53
C_2	6.77	8.13	0.6	128.81	164.06	0.27

*SOM = soil organic matter

Table 3

Laboratory data of the topsoil in Gömörszőlős

Surface cover	Slope	pH_{KCl}	pH_{H_2O}	$CaCO_3$ (%)	AL-P_2O_5 (mg kg^{-1})	AL-K_2O (mg kg^{-1})	SOM*** (%)
Arable land	Upper*	6.68	7.78	21.3	140.84	463.99	2.33
	Lower**	6.81	7.77	7.8	166.36	558.55	3.16
Grassland	Upper	6.71	7.33	19.3	110.14	483.00	3.91
	Lower	6.63	7.16	9.7	181.60	532.20	4.45

*Upper = upper third of the slope, **Lower = lower third of the slope, ***SOM = soil organic matter

In the Alsószuha area, the basic soil parameters of an arable area were compared with those of plots abandoned 10 and 42 years ago and now mostly used for haymaking. As can be seen in Table 4, there were differences in the distribution of the examined soil parameters.

Coenological data on the plant taxa and cover percentages are presented in Tables 5–7. All the plots were previously sown to maize (*Zea mays*), winter wheat (*Triticum aestivum*) or oats (*Avena sativa*).

The first list (Table 5) was prepared for the plot that has been used as a hay meadow for approx. 10 years and was previously used to grow maize and alfalfa. The highest number of plant species was recorded on this plot, probably due to regular mowing, as the removal of biomass from the area may result in the appearance of certain weed species. This plot was oversown with red clover (*Trifolium pratense*). The relatively high rate of dicotyledonous plant species is indicative of a balanced, fairly stable sward.

Table 4
Laboratory data of the topsoil in Alsószuha

Surface cover	Slope	pH_{KCl}	pH_{H_2O}	$CaCO_3$ (%)	AL-P_2O_5 (mg kg^{-1})	AL-K_2O (mg kg^{-1})	SOM*** (%)
Arable land	Upper*	5.41	6.50	0	32.41	162.68	2.55
	Lower**	5.96	6.70	0	90.07	184.35	3.28
Abandoned (10 years	Upper	5.32	6.30	0	28.67	141.86	3.01
	Lower	5.25	6.16	0	20.85	118.72	2.37
Abandoned (40 years)	Upper	6.47	6.85	0	66.59	166.23	2.50
	Lower	5.70	6.37	0	19.58	188.04	2.86

*Upper = upper third of the slope, **Lower = lower third of the slope, ***SOM = soil organic matter

Table 5
Coenological data on a 10-year-old hay meadow in Alsószuha

Species	Cover (%)	Species	Cover (%)
Achillea collina	5	*Lotus corniculatus*	2
Agrimonia eupatoria	1	*Pastinaca sativa*	4
Anagallis arvensis	1	*Picris hieracioides*	1
Artemisia vulgaris	2	*Plantago lanceolata*	3
Calamagrostis epigeios	2	*Plantago media*	5
Centaurea macroptilon	15	*Poa angustifolia*	10
Chrysanthemum leucanthemum	1	*Prunella vulgaris*	3
Cichorium intybus	5	*Ranunculus polyanthemos*	2
Convolvulus arvensis	1	*Setaria glauca*	5
Coronilla varia	3	*Symphytum officinale*	3
Dactylis glomerata	2	*Taraxacum officinale*	5
Daucus carota	3	*Tragopogon orientale*	2
Hypericum perforatum	1–2	*Trifolium pratense*	15
Inula britannica	3	*Trifolium repens*	8
Leontodon hispidus	2	*Verbascum blattaria*	1
Linaria vulgaris	1-2		

The species in the second list (Table 6) were detected in the neighbouring plot, on which cereal production was discontinued approx. 10 years ago, and which is currently more weedy due to the lack of regular mowing. The high (40%) cover of the aggressive weed *Elymus repens* reflects the disturbed state of the land and confirms the lack of regular mowing. The relatively short time that has elapsed since the cereal culture was abandoned was not sufficient for revegetation from nearby natural grasslands, and weed species are dominant because of the poor management. Invasive weeds, however, are absent. The total number of species is also less than on the first plot.

The third list (Table 7) shows the species on the third plot, which was abandoned in 1963 and grazed by sheep until 1990. It is currently uncultivated and in a close-to-natural state. More species were found than on the second plot, but less than on the first. The dominance of *Brachypodium pinnatum* shows that over the last 43 years a secondary grassland (seeded by species from neighbouring patches of natural vegetation) has developed, forming a balanced habitat (slope steppe) with wildflower species in a close-to-natural state. Weed species characteristic of arable land (*Anagallis arvensis, Equisetum arvense, Setaria glauca*) are rare and there are no invasive weeds. The coenological data reflect the fact that the plot was previously used for grazing, as both grass species with great forage value (*Agrostis stolonifera, Brachypodium pinnatum, Festuca rupicola*) (Benyovszky et al., 1995) and weed species typical of pastures (*Eryngium campestre*) were found. *Achillea collina* and *Galium verum* can also be considered as indicators of grazing.

Discussion

The results show that maize cannot provide sufficient protection against phosphorus loss. Grasslands usually gave better results, but it was still perceptible if the land was used for arable farming before being turned into grassland (hay meadows).

Table 6
Coenological data on a plot abandoned and not regularly mowed in Alsószuha

Species	Cover (%)	Species	Cover (%)
Anagallis arvensis	1	*Matricaria inodora*	2
Bromus arvensis	2	*Mentha longifolia*	4
Cichorium intybus	3	*Pastinaca sativa*	2
Cirsium arvense	5	*Setaria glauca*	5
Convolvulus arvensis	3	*Stenactis annua*	4
Daucus carota	1–2	*Symphytum officinale*	2
Elymus repens	40	*Taraxacum officinale*	5
Lathyrus tuber	1	*Trifolium repens*	3
Leontodon hispidus	2		

Table 7
Coenological data on a plot abandoned in 1963 and grazed until 1990 in Alsószuha

Species	Cover (%)	Species	Cover (%)
Achillea collina	2	*Hieracium umbellatum*	3
Agrimonia eupatoria	10	*Knautia arvensis*	2
Agrostis stolonifera	5	*Leontodon hispidus*	4
Anagallis arvensis	1	*Linum catharticum*	2
Brachypodium pinnatum	25	*Lotus corniculatus*	2
Calamintha vulgaris	1	*Ononis arvensis*	2
Cerastium vulgatum	1	*Pimpinella saxifrage*	2
Dorycnium germanicum	5	*Plantago media*	3
Equisetum arvense	1	*Ranunculus polyanthemos*	2
Eryngium campestre	2	*Setaria glauca*	3
Festuca rupicola	5	*Thesium linophyllon*	2
Galium verum	3	*Trifolium pratense*	1

Based on laboratory investigations on various slope angles and soil types (and/or soil characteristics, such as soil structure, soil organic matter content, water management, etc.) recommendations can be made to help farmers choose the necessary measures to protect their land from soil and fertilizer loss.

The composition of plant associations and habitats may differ significantly, depending on the current and previous land use. It is important to know the effects of various types of land use in order to determine the nature protection and economic value of these land use methods.

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EFFECT OF LOW DOSE UV-C RADIATION ON THE GERMINATION RATE AND FUNGAL CONTAMINATION OF TALL FESCUE (*Festuca arundinacea* SCHREB.) SEEDS

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The effects of UV-C radiation on the germination rate and fungal contamination of tall fescue seeds were investigated. Samples from the same seed lot were irradiated in two different ways in two consecutive years. The seeds were irradiated with a Hg vapour lamp using different doses. In the first trial one side of the seeds was irradiated, while in the second trial a mirror was used to irradiate the whole seed surface. The results showed that various doses of UV-C irradiation had an effect on the germination rate, but there were no significant differences in germination percentage between the treatments. Differences in fungal contamination rates were observed when the seeds were irradiated on all sides. The presence of 21 fungal genera was identified on the seeds, the saprotrophic fungi *Alternaria, Rhizopus* and *Penicillium* being dominant.

Key words: tall fescue, UV-C irradiation, germination, fungal contamination, *Alternaria*

Introduction

Tall fescue (*Festuca arundinacea* Schreb.) is a loosely tillering perennial grass species. It has been naturalized all over the world, and in Australia and New Zealand it is one of the most important forage plants (Easton et al., 1994). It is used as a basic mass forage for beef cattle breeding, because of the large fresh mass. It starts to grow early in spring, allowing earlier grazing, which can be continued until late autumn, even at a temperature of –4°C.

A healthy, dense crop can only develop from disease-free, well-germinating seeds. As chemicals may pollute the environment, another alternative has to be found to disinfect seeds. One such possibility could be the irradiation of seeds with UV-C radiation.

In Hungary, Nagy and Fischl (2003) investigated the effects of UV-C irradiation on the germination of the conidia of *Alternaria alternata* and *Curvularia inaequalis* and found that the semi-lethal dose was 5.5 J/cm^2 for *Alternaria alternata* conidia and 0.043 J/cm^2 for *Curvularia inaequalis*. Nagy et al. (2005) investigated the effect of UV-C irradiation on *Fusarium culmorum* cultures and on conidium germination and showed that the conidia developing on *Fusarium culmorum* cultures irradiated with a 0.81 J/cm^2 dose had higher percentage germination than that of the controls, while conidia irradiated with a higher dose did not germinate.

The effect of UV radiation and visible light on polyphagous plant pathogenic fungi was investigated in the case of *Sclerotinia sclerotiorum* (Nagy and Fischl, 2002a) and *Macrophomina phaseolina* (Nagy and Fischl, 2002b), and UV irradiation was found to inhibit the mycelium growth of both fungi. On the other hand, Kumagai (1978) found that near UV irradiation stimulated the sporulation of *Drechslera oryzea* and *Botrytis cinerea*. According to Nigro et al. (1998) stored dessert grapes irradiated with UV-C exhibited less fruit rot caused by *Botrytis cinerea*. Stevens et al. (1996; 1998) observed the useful effects of UV-C irradiation against fungal pathogens causing rot in various fruit species. Marquenie et al. (2002) applied UV-C and heat treatment, and found that *Monilinia fructigena* reacted more sensitively to these treatments than *Botrytis cinerea*.

Brown et al. (2001) treated cabbage seeds with low dose UV-C radiation against the black-vein disease of cabbage species (*Xanthomonas campestris* pv. *campestris*) and found that treating seeds with a dose of 0.36 J/cm^2 considerably decreased the occurrence of the disease. Nolasco et al. (1996) tested the germination of seeds of the giant cardón cactus (*Pachycereus pringlei*) following various treatments, including UV irradiation. There were no significant differences in germination between the treated and control seed lots.

The aim of the present work was to investigate the germination ability of tall fescue and the occurrence of seed-contaminating fungal pathogens after treatment with various doses of UV-C irradiation.

Materials and methods

The investigations were made in January and February 2005 and 2006, using seeds of the tall fescue cultivar Strand. The seed sample was harvested in 2004 and stored in a well-aerated frost-free room in paper bags. UV radiation was produced using a 125 W Hg vapour lamp, which radiates mainly at a wavelength of 254 nm. Four replications of 100 seeds were irradiated for 15, 60, 120, 180, 240, 260 and 300 minutes with an intensity of 0.9 mW/cm^2 UV light, giving irradiation doses of 0.81, 3.24, 6.48, 9.72, 12.96, 14.04 and 16.2 J/cm^2, respectively. In 2005 the seeds were irradiated only from above, with doses of 0.81, 3.24, 6.48, 9.72, 14.04 and 16.2 J/cm^2, while in 2006 a mirror was used to reflect the light onto the other surfaces, with doses of 0.81, 3.24, 6.48, 9.72, 12.96 and 16.2 J/cm^2. The distance between the lamp and the seeds was 60 cm, with 20 cm between the seeds and the mirror. The intensity of reflected light was estimated to be about 90% of the direct light intensity. Following treatment, the seeds were layered on wet double filter papers in Petri dishes, previously sterilized at 60°C for 3 h to avoid contamination. The germination test was carried out according to the Hungarian standards (MSZ 6354/3, MSZ 6354/5) and ISTA regulations in natural light at room temperature using seeds without surface sterilization. During the determination of germination rate and germination vigour, mycological assessments were made on the seventh and fourteenth days. A stereo-microscope (×20–45) and a binocular light microscope (×64–1000) were used to determine the rate of fungal contamination and the morphological and size characteristics of the fungal species contaminating the seeds. To ensure accurate identification, intact cultures were also produced in some cases. Digital photographs of the pathogens were taken and the descriptions given by Radulescu and Negru (1971), Ellis (1971), Sutton (1980) and Hanlin (1990) were used to identify the fungal species.

The SPSS 9.0 software was used for statistical analysis, involving single factor analysis of variance and linear regression analysis. The germination rate and fungal infection rate of seed lots given different doses of radiation were compared to the values of the non-irradiated (0 J/cm^2) seed lot, measured at the same time. Arcsin transformation was carried out before the analysis of variance. The level of significance was $p<0.05$. The effect of UV-C radiation on the germination rate and fungal infection rate of the treated seeds was investigated by linear regression analysis.

Results and discussion

Effect of UV-C irradiation on germination

In 2005 the germination vigour of seed samples irradiated with doses of 6.48, 14.04 and 16.2 J/cm^2 was significantly ($p<0.05$) higher than that of the non-irradiated control (Fig. 1). In 2006 the results were similar (Fig. 2). At the first observation date (7th day) the germination vigour was significantly higher for the highest doses (12.96 and 16.2 J/cm^2) than in the control. The 0.81 J/cm^2 treatment also differed significantly from the 2005 result. It should be noted that although the seed sample was a year older in 2006, its germination vigour was about 10% higher than in 2005.

The germination percentage of the non-treated seeds was nearly the same in both years (94.75 and 94.25%), and similar results were obtained for samples treated with various doses of UV-C light. They germinated well, and no significant differences were observed between the different doses of irradiation (Figs. 1 and 2). Nolasco et al. (1996) did not observe significant differences in the germination of giant cardón cactus seeds irradiated with various doses of UV-C radiation.

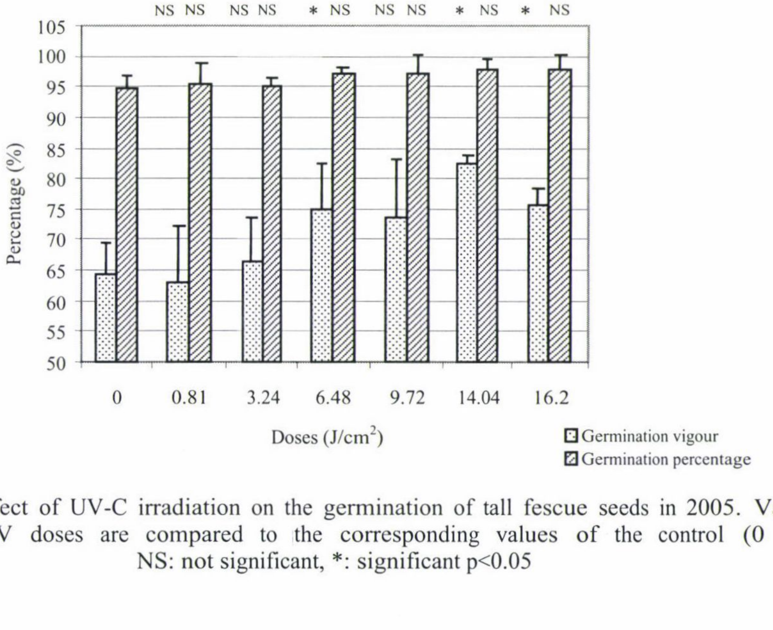

Fig. 1. Effect of UV-C irradiation on the germination of tall fescue seeds in 2005. Values at various UV doses are compared to the corresponding values of the control (0 J/cm^2). NS: not significant, *: significant $p<0.05$

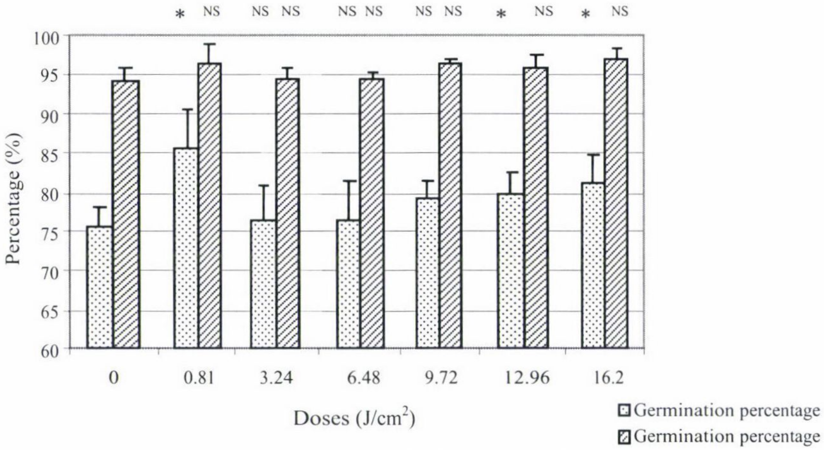

Fig. 2. Effect of UV-C irradiation on the germination of tall fescue seeds in 2006. Values at various UV doses are compared to the corresponding values of the control (0 J/cm^2). NS: not significant, *: significant $p<0.05$

Effect of UV-C irradiation on the fungal contamination rate of seeds

A high rate of fungal contamination was observed on the seeds in 2005. A slight decrease in the contamination rate could be observed for the UV-C treated samples, but it was only significant for the 14.04 J/cm^2 treatment. This treatment was also significantly less contaminated at the time of the second observation, while the 16.2 J/cm^2 treatment showed a significantly higher value than that of the control sample (Fig. 3). It should be noted that in this case the irradiation was given only from above.

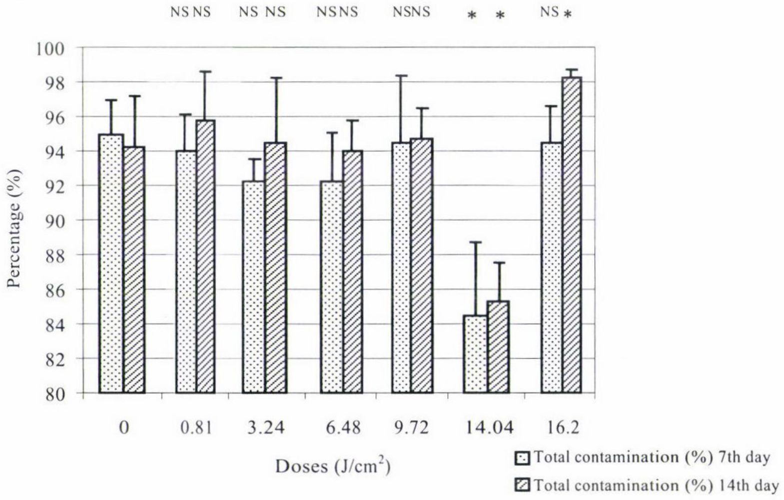

Fig. 3. Changes in fungal contamination rates of tall fescue seeds following UV-C irradiation in 2005. Values at various UV doses are compared to the corresponding values of the control (0 J/cm^2). NS: not significant, *: significant $p<0.05$

It is interesting to note that at the time of the first observation in 2006 the fungal contamination rate of non-treated seeds was 59% lower than in 2005. At the time of the second observation this value had fallen to 47%. The fungal contamination rate in the 6.48, 9.72, 12.96 and 16.2 J/cm^2 treatments was significantly lower than that of the control at the first observation date. At the second observation date the fungal contamination rate of all the treated samples was significantly lower than that of the non-treated samples (Fig. 4). Based on linear regression analysis it can be concluded that the different UV doses correlated positively with the germination rate and negatively with the fungal infection rate (see Table 1).

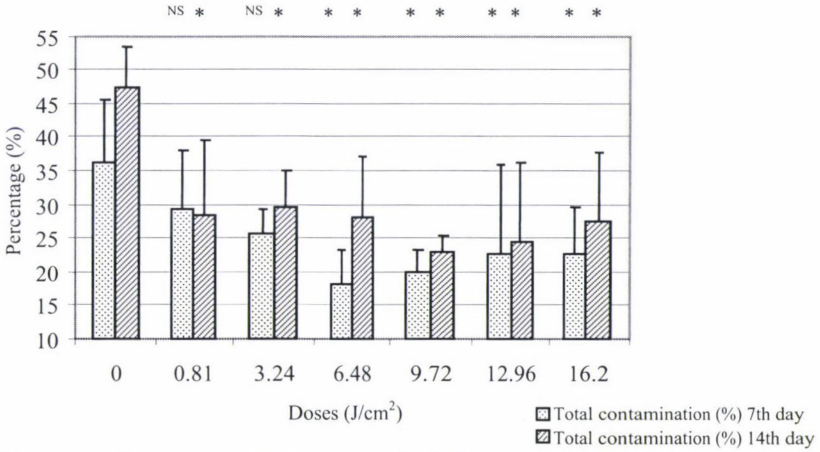

Fig. 4. Changes in fungal contamination rates of tall fescue seeds following UV-C irradiation in 2006. Values at various UV doses are compared to the corresponding values of the control (0 J/cm^2). NS: not significant, *: significant $p<0.05$

During the tests a large number of fungal species could be identified on the seed surfaces, belonging to 21 fungal genera, mainly to the *Deuteromycota*, but with some *Ascomycota*. Saprotrophic fungi, such as *Alternaria* spp., *Rhizopus stolonifer* and *Penicillium* spp., were dominant. Ninety per cent of the contamination could be attributed to the *Alternaria* genus in 2005 and 50–70% in 2006. Walcz and Horváth (1976) found that 35–65% of the total contamination on the seeds of different grasses was due to *Alternaria alternata*. In the present tests the rate of contamination with *Rhizopus stolonifer* was similar in the two years, while the occurrence of the *Penicillium* species was very low in 2005, but made up about 10% of the total fungal contamination in 2006. During the two years of the tests the following fungal genera or species were also identified at low frequency: *Pyrenophora* sp., *Sordaria fimicola*, *Chaetomium* spp., *Phoma epicoccina*, *Septoria* sp., *Aureobasidium pullulans*, *Aspergillus* spp., *Acremoniella atra*, *Epicoccum nigrum*, *Cladosporium* spp., *Stemphylium botryosum*, *Arthrinium sphaerospermum*, *Embellisia* sp., *Embellisia dennisii*, *Ulocladium* sp., *Gonatobotrys flava*, *Drechslera* spp., *Bipolaris spicifera* and *Bipolaris sorokiniana*. It is interesting to note that no species of *Fusarium* were detected.

Table 1
Results of regression analysis as a function of UV doses

Year		b	a	r
2005	Germination vigour (7th day)	64.23^{**}	0.998^{**}	0.664^{**}
	Germination rate % (14th day)	94.97^{**}	0.213^{**}	0.521^{**}
	Fungal infection rate (7th day)	94.12^{**}	–0.23	–0.323
	Fungal infection rate (14th day)	94.85^{**}	–0.143	–0.194
2006	Germination vigour (7th day)	78.62^{**}	0.09	0.112
	Germination rate % (14th day)	94.8^{**}	0.115^{*}	0.392^{*}
	Fungal infection rate (7th day)	29.66^{**}	-0.685^{*}	-0.439^{*}
	Fungal infection rate (14th day)	35.34^{**}	-0.812^{*}	-0.435^{*}

Note: *: $p<0.05$; **: $p<0.01$. a: slope, b: intersection

On the basis of the results it can be stated that irradiation with a dose of above 12 J/cm^2 had an effect on the initiation of germination in tall fescue seeds, but had no influence on the germination rate. The germination rates of one- and two-year-old seeds were similar. Changes were observed in the fungal contamination rate of the seeds and in the dominance relationships of the contaminating fungi. The irradiation of the seeds from all sides at a dose of higher than 3.24 J/cm^2 considerably decreased the fungal contamination rate. The UV-C irradiation of seeds may be an effective method for practical use, but further investigations will be needed. It should also be mentioned that while the rate of fungal contamination decreased following UV-C treatment at a dose higher than 3.24 J/cm^2, an increase in germination vigour was also observed.

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EVALUATION OF THE APICULTURAL VALUE OF SUNFLOWER HYBRIDS

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Sunflower is one of the most important bee-pasture crops and the leading oil crop plant in Hungary. There are very few studies concerning the nectar production of the plant, most of which consist only of partial data that show the apicultural value of sunflower under intensive cultivation conditions.

The nectar production and nectar sugar concentration of six sunflower hybrids, Arena, Alexandra, Cledor, Coriste, Hysun 321 PR and Louidor, were examined in Mezőhegyes (south-east Hungary) from 2002 to 2004. The aim was to determine the nectar production and overall apicultural value of the hybrids. In the experiment the agroecological conditions were also examined and recorded. These agroecological conditions showed a distinctive effect on the consistency of the apicultural values of the hybrids.

It can be determined from the results that the nectar production and its sugar content can be modified measurably by external factors. The nectar quantity was measurably increased by abundant precipitation during flowering, while an increase in the nectar sugar content was caused by excessively low air temperature. During these three years the average nectar production of the hybrids was 0.147 mg/floret, with a sugar content of 48.8%. Significant differences were found between the hybrids in nectar production and in the nectar sugar concentration.

Averaged over three years Coriste displayed the best apicultural value. Its nectar production was stable and high (0.167 mg/floret). Its high sugar content (49.1%) also proved to be attractive to honey bees (sugar value 0.082). The lowest apicultural value was displayed by the hybrid Alexandra, with a sugar value of 0.059. This suggests that the honey production value of the individual hybrids should be taken into consideration during the selection of bee pastures.

Key words: apicultural value, sunflower, nectar production, sugar concentration

Introduction

Sunflower is the leading oil crop plant in Hungary, but it is also highly valued in apiculture as a summer bee pasture (Farkas, 1983; Ruff, 1991). Its flowering period lasts throughout July and August (Nyárády, 1958) when very few other nectar plants are available for honey bees. Therefore, throughout the summer, sunflower is a significant bee pasture that ensures long honey flow, which can be partially attributed to its wide-scale production. The wide range of sunflower hybrids has also generated many questions regarding their apicultural value. From the crop producers' point of view the main aim is to achieve the highest possible yield and oil production. From the beekeepers' point of view the most important is the apicultural value of the hybrids. It is very difficult to choose hybrids well suited to the ecological conditions, and satisfying the criteria of both crop producers (Pepó and Borbélyné, 2003) and beekeepers. Beekeepers generally have to be satisfied with oil-type hybrids, in which the nectar production may vary.

Similarly, determining the nectar production of a crop in a given country raises several problems, because changes in external factors may alter the results significantly.

A few facts need to be stated in order to understand the relationship between sunflowers and bees. On the one hand, sunflowers need bee pollination (Free, 1970), on the other hand, the nectar and pollen of the plant are very attractive to insects, particularly to honey-bees (Free and Simpson, 1964; Frank and Kurnik, 1970). They gather nectar mainly in sunflower fields (Benedek and Manninger, 1972).

According to Benedek et al. (1974) the extent to which honey bees are attracted depends on the volume of nectar and its sugar concentration, which vary from one hybrid to the other (Lajkó, 2002). Nectar production is influenced not only by genetic factors (Huber, 1956; Kamler, 1997; Lajkó, 2002), but also by ecological factors (Halmágyi and Suhayda, 1963; Gulyás, 1975; Pesti, 1980; Gulyás and Frank, 1995; Hedtke, 1998; Zajácz et al., 2002).

Temperature and air humidity are the main factors that influence the nectar volume and sugar concentration within the sunflower plants themselves (Pesti, 1980). In the investigations of Péter (1983) the flowers produced only a small amount of nectar due to the hot and arid weather conditions that occurred throughout the summer season. In the experiment of Halmágyi and Suhayda (1963) the florets had high nectar production (0.405–0.487 mg/floret) with 49.5–51.3% sugar concentration. In the measurements of Mészáros and Gulyás (1994) the hybrids produced 0.05–0.26 mg nectar, but the average concentration was very low (25%).

The low values of nectar in recent years were caused by high temperature and sandy soils according to Hedtke (1998). Koltowski (2006) reported 3.14 to 7.19 mg total sugar quantity from 10 blossoms of sunflower hybrid PR64A54 on individual days of the investigations.

The aim of this study was to investigate the nectar-producing ability of the sunflower hybrids currently grown in Hungary in order to determine their apicultural importance. The influence of environmental factors on nectar volume and concentration was also examined.

Materials and methods

The nectar production and nectar sugar concentration of six sunflower hybrids, Arena, Alexandra, Cledor, Coriste, Hysun 321 PR and Louidor, were examined in Mezőhegyes from 2002 to 2004. The experimental field had meadow chernozem soil (pH=7) with a humus content of 4% and surface soil to a depth of 1 m.

Samples were taken from randomly chosen plants after 24 hours of isolation. Fifty disc florets per head were examined in each year of the study. Nectar sampling took place over five days in four replications per day, i.e. from four heads of each hybrid, in 2002 and 2004 and in three replications per day in 2003. Glass capillaries were used for measuring the nectar production. The sugar concentration of the nectar was determined with a refractometer. The sugar value was calculated from the amount of nectar and its refraction value. Microclimatic parameters such as temperature and air humidity were measured hourly.

The data were statistically analysed by analysis of variance at the $P \leq 0.02$ and $P \leq 0.03$ levels.

Microclimatic parameters

Large amounts of precipitation were recorded during the 2002 and 2004 growing seasons (341 mm and 426 mm, respectively), mainly in June and July. It rained twice during the flowering period in 2002. Otherwise, the weather was ideal for the measurements and for flowering periods of sunflower in 2002 and 2004. In 2003 the growing season was very dry, with a total rainfall of only 125 mm during the growing season, which is exceptionally low, leading to drought stress in June and July (Table 1).

Results

Nectar production

The average nectar production of the hybrids in the three years of the study is shown in Table 2. There was a statistically significant difference ($P \leq 0.03$) in the nectar production of the individual hybrids, as also reported by Kamler (1997). The highest nectar production was recorded in 2002, when it rained twice during the sampling period. The average air temperature was the lowest and the average humidity the highest of all the years monitored. The Arena and Cledor hybrids produced the highest nectar amount, whereas the lowest amounts were found for Alexandra and Hysun 321 PR. The growing season in 2003 was hot and arid and severely influenced nectar secretion. The weather became unfavourably cloudy and windy several times, and resulted in lower but more concentrated nectar production.

The average nectar amount in 2003 was 0.138 mg/floret and only two hybrids, Coriste and Cledor, secreted more nectar than the average. The abundant precipitation during the growing period in 2004 balanced the unfavourable weather witnessed throughout the sampling period. Higher quantities of nectar were recorded in 2004 than in 2003 due to the precipitation experienced twice during the measurements (Table 2).

Table 1
Average temperature, air humidity and precipitation during the experiments in Mezőhegyes (2002–2004)

Year	Temperature, °C			Air humidity, %			Average precipitation during the growing season, mm
	Min.	Max.	Average	Min.	Max.	Average	
2002	15	38	25	25	85	59	341
2003	17	38	27	34	86	52	125
2004	16	39	28	28	60	42	426

Table 2
Average nectar production of the hybrids in Mezőhegyes (2002–2004)

Hybrid	Nectar production mg/floret		
	2002 $\bar{x} \pm s$	2003 $\bar{x} \pm s$	2004 $\bar{x} \pm s$
1. Arena	0.182 ± 0.027 c	0.130 ± 0.033 ad	0.148 ± 0.023 ac
2. Alexandra	0.145 ± 0.034 b	0.108 ± 0.021 c	0.127 ± 0.038 bd
3. Cledor	0.171 ± 0.043 ac	0.147 ± 0.049 ad	0.149 ± 0.032 ad
4. Coriste	0.149 ± 0.026 ab	0.190 ± 0.059 b	0.162 ± 0.027 a
5. Hysun 321 PR	0.143 ± 0.028 b	0.127 ± 0.045 ac	0.164 ± 0.046 a
6. Louidor	0.157 ± 0.043 ab	0.122 ± 0.027 cd	0.132 ± 0.045 bcd
Average	0.158 ± 0.016	0.138 ± 0.029	0.147 ± 0.015

Values designated with different letters are significant at the $P \leq 0.03$ level.

Sugar concentration of the nectar

Table 3 shows the average refraction values of the hybrids, ranging from 38.3–63.8%. In 2002 the average sugar concentration of the nectar was 39.8%, which was the lowest concentration recorded over the three-year testing period. It should be noted that the nectar was the most diluted in 2002, when the nectar amounts were the highest.

The most concentrated nectar was measured in 2004, when the average refraction values ranged from 55.7–63.8%, making it more attractive to honeybees, but the foraging activities of the honeybees were less intensive due to the high air temperatures.

Summarizing the data of the three years of study, the highest nectar concentration was measured in the hybrid Cledor (63.8%) in 2004, while the lowest value was observed in Coriste (38.3%) in 2002.

There was a significant difference ($P \leq 0.02$) in the sugar concentration of the nectar in different hybrids in the same year (Table 3).

Sugar value

The sugar values of the hybrids, indicating their apicultural value, are shown in Table 4. They were similar in 2002 and 2003, enabling the hybrids to be ranked. The highest sugar value (0.088 averaged over all the hybrids) was measured in 2004, due to the higher sugar concentrations. Figure 1 shows the total sugar value of the hybrids averaged over three years. The lowest sugar value was obtained for Alexandra and the highest for Coriste (Table 4, Figure 1).

Table 3
Average sugar concentration of the nectar in Mezőhegyes (2002–2004)

Hybrid	Sugar concentration %		
	2002 $\bar{x} \pm s$	2003 $\bar{x} \pm s$	2004 $\bar{x} \pm s$
1. Arena	38.5 ± 4.0 b	49.5 ± 7.5 a	58.0 ± 3.6 bc
2. Alexandra	39.1 ± 3.5 b	43.9 ± 6.7 bc	55.7 ± 4,9 bcd
3. Cledor	38.9 ± 3.0 b	50.1 ± 8.4 a	63.8 ± 2.7 a
4. Coriste	38.3 ± 2.5 b	46.7 ± 5.9 ac	62.2 ± 2.4 a
5. Hysun 321 PR	38.9 ± 1.6 b	46.4 ± 4.1 ac	59.7 ± 3.3 b
6. Louidor	44.9 ± 5.7 a	45.8 ± 12.2 ac	57.1 ± 5.7 b
Average	39.8 ± 2.5	47.1 ± 2.3	59.4 ± 3.1

Values designated with different letters are significant at the $P \leq 0.03$ level.

Table 4
Nectar sugar value of the hybrids in Mezőhegyes (2002–2004)

Hybrid	Sugar value, mg/floret		
	2002 $\bar{x} \pm s$	2003 $\bar{x} \pm s$	2004 $\bar{x} \pm s$
1. Arena	0.071	0.064	0.086
2. Alexandra	0.057	0.048	0.071
3. Cledor	0.067	0.072	0.095
4. Coriste	0.057	0.088	0.101
5. Hysun 321 PR	0.056	0.059	0.098
6. Louidor	0.072	0.055	0.075
Average	0.063±0.007	0.064±0.014	0.088±0.012

Discussion

It can be concluded that, averaged over three years, the hybrid Coriste was the most attractive to honeybees with its high volume of concentrated nectar; this was reflected in the ranking of the hybrids on the basis of nectar sugar value. Louidor and Alexandra had the lowest values, both in terms of nectar amount and concentration. Significant differences were observed in the quantity and sugar concentration of the nectar in different hybrids in the same year and also over the three-year period.

The average nectar production of sunflower florets (0.138–0.158 mg nectar/floret) was similar to the values reported by Mészáros and Gulyás (1994) (0.05–0.26 mg), but lower than those measured by Halmágyi and Suhayda (1963) (0.405–0.487 mg), Du Toit and Coetzer (1991) (0.912–1.656 mg/floret), Kamler (1997) (17.7 mg nectar/50 flowers on a 3-year average) and Koltowski (2006) (0.314–0.719 mg). It should be noted, however, that none of the previous studies investigated the hybrids used in the present study. This can be attributed to the large number of sunflower hybrids commonly grown in Hungary. The differences in nectar production may also be related to the varied growing conditions (e.g. field or greenhouse cultivation, differences in soil type and moisture) and sampling methods.

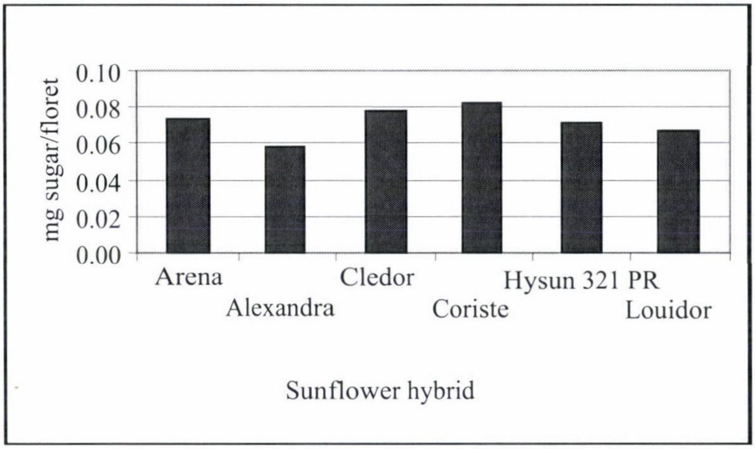

Fig. 1. Sugar value of the hybrids, averaged over 3 years in Mezőhegyes (2002–2004)

The nectar production values in the present study were primarily influenced by microclimatic conditions, especially by the volume of precipitation and temperature. The nectar was also more concentrated at low relative humidity, as described by Schuel (1952).

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DIRECT AND RESIDUAL EFFECTS OF AGRO-INDUSTRIAL WASTES ON A ROCKET SALAD (*Eruca sativa* Mill.) – SORGHUM [*Sorghum bicolor* (L.) Moench] SEQUENCE

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A field study conducted for two years (2002–04) at New Delhi showed that the seed yield (1.80 t ha^{-1}) of rocket salad (*Eruca sativa* Mill.) obtained by applying 5 t ha^{-1} pressmud compost based on distillery effluent + half the recommended dose of NPKS (recommended dose: 60 kg N, 13 kg P, 25 kg K and 20 kg S ha^{-1}) was on par with the seed yield (1.69 t ha^{-1}) recorded with the recommended dose of NPKS. However, the seed yield recorded with the former treatment significantly exceeded that obtained with 5 t ha^{-1} of a 1:1 mixture of fly ash and distillery effluent + half the recommended dose of NPKS (by 30.4%) or 5 t ha^{-1} of dry *Jatropha curcas* leaves + ½ NPKS (by 24.1%). On average, distillery effluent-based pressmud compost + ½ NPKS induced a perceptible increase in the soil-available NPK, recorded after the harvest of rocket salad, compared to the initial fertility status. The uptake of NPKS in the seed and stover of rocket salad was the highest after the application of pressmud compost, closely followed by the recommended dose of NPKS, and the lowest in the control. The residual effect of treatments given to rocket salad was significant on the fodder yield of succeeding sorghum [*Sorghum bicolor* (L.) Moench]. The fodder yield recorded with pressmud compost + ½ NPKS was significantly higher than the other treatments. The application of pressmud compost alone was also significantly superior to the same rate of fly ash + effluent mixture or dry *Jatropha* leaves with respect to the seed yield of rocket salad, residual fertility after the harvest of rocket salad and the fodder yield of succeeding sorghum.

Key words: rocket salad, *Eruca sativa*, distillery effluent-based pressmud compost, fly ash + distillery effluent mixture, dry *Jatropha* leaves, sorghum, residual fertility

Introduction

Rocket salad (*Eruca sativa* Mill.) is an annual oilseed crop grown under limited soil moisture conditions. In Europe, certain ecotypes of rocket salad are generally grown for their young leaves, which are eaten as green salad, whereas in India and other Asian and African countries, it is mainly grown for oil production. It has potential to outyield Indian mustard (*Brassica juncea*) and chickpea (*Cicer arietinum*) under moisture stress conditions (Divakara Sastry, 2003). Integrating chemical fertilizers with organic manures has been found to be promising not only in maintaining high productivity but also in providing greater stability in crop production (Ramesh et al., 2006). Farmyard manure is used as a major source of organic nutrients in field crops. The limited availability of this manure due to alternate uses is an important constraint on its use, stressing the need for the evaluation of other locally available agro-industrial waste materials as a substitute for farmyard manure and chemical fertilizers.

In India the sugar industry generates about 5–6 million tons of pressmud and 7.5 million tons of molasses as by-products annually. Molasses is utilized for industrial alcohol production and pressmud is used for compost preparation. In India, molasses-based distilleries face a severe problem in disposing their anaerobically digested, foul-smelling, discoloured wastewater, which is generated in large quantities, and has a very high biological and chemical oxygen demand (5000–8000 mg L^{-1} and 25,000–30,000 mg L^{-1}, respectively) (Joshi et al., 1996). Its injudicious discharge on land surfaces and in watercourses damages aquatic life and impairs soil and groundwater quality. However, this distillery wastewater contains an appreciable amount of macro- and micronutrients (organic carbon, K, N, Fe, Cu, Zn and B, etc.), which are essential for plant growth. Several workers have suggested its use in agriculture, either as a soil amendment without dilution before crop sowing or in conjunction with irrigation water for application to standing crops (Pathak et al., 1999). Pressmud, which is also generated in large quantities by the sugar industry, has good manure potential. Many distilleries have now started making compost out of pressmud by mixing it with distillery effluent along with microbial inoculation. Another waste generated from distilleries is fly ash, which also causes disposal problems in and around the area of the distillery. However, fly ash also contains substantial amounts of plant nutrients along with other components. The incorporation of a fly ash + distillery effluent mixture into the soil modifies the soil environment, especially its moisture retention and physicochemical properties, thus promoting root-shoot growth and grain yield in wheat (Kalra et al., 1998; Adriano and Weber, 2001). The proper utilization of agro-industrial wastes in agriculture not only reduces dependence on chemical fertilizers but also minimizes the environmental problems caused by these industrial waste materials if disposed non-judiciously.

On the other hand the use of industrial wastes in agricultural production has also been reported to increase the concentration of heavy metals in the soil (Gupta et al., 1986; Petruzzelli, 1989; Maiti et al., 2003; Faheed, 2005). Industrial wastes have a residual effect in the soil, so studies are needed in succeeding crops. After rocket salad, a short duration crop of summer fodder sorghum is a good choice under the limited irrigation water conditions of the semi-arid tropics in order to sustain the livestock during the summer. Information on the use of agro-industrial waste materials in crop production under field conditions is very limited; moreover, the actual benefits of these materials depend upon many factors. So an attempt was made to find out the effect of these agro-based industrial wastes on crops and soil, and how they can best be integrated with chemical fertilizers.

Materials and methods

Site and soil

A field experiment on a rocket salad–sorghum cropping system was conducted over two years (2002–03 and 2003–04) at the experimental farm of the Indian Agricultural Research Institute, New Delhi, India. The site is located in the Indo-Gangetic alluvial tract at 28°40' N and 77°12' E, at an altitude of 228 m above mean sea level. The area receives an annual rainfall of 652 mm, about 80% of which occurs from June to September. The mean maximum and minimum temperatures are 35 and 18°C from July to October and 22.6 and 6.7°C from November to April. The alluvial soil of the experimental site was sandy loam in texture (46% sand, 33% silt and 21% clay) and had a bulk density of 1.38 g cm^{-3}, a pH (1:2 soil : water) of 8.1, an electrical conductivity of 0.48 dS m^{-1}, and a CEC of 7.3 C mol (p^{+}) kg ha^{-1}, while the available N, P and K were 215, 13.2 and 205 kg ha^{-1}, respectively.

Treatments

Rocket salad received treatments during the winter season (October–March) while sorghum was grown on residual fertility during the summer (April–June). The experiment was conducted in a randomized block design having three replications. The eight treatment combinations of agro-industrial wastes, dry *Jatropha* leaves and NPKS from fertilizers were as follows:

1. Control
2. Recommended dose of NPKS (60 kg N, 13 kg P, 25 kg K and 20 kg S ha^{-1}), code: NPKS
3. 5 t ha^{-1} of a 1:1 mixture of fly ash and distillery effluent, code: fly ash
4. 5 t ha^{-1} of pressmud compost based on distillery effluent, code: pressmud
5. 5 t ha^{-1} of dry *Jatropha* leaves, code: Jatropha
6. 5 t ha^{-1} of a 1:1 mixture of fly ash and distillery effluent + half the recommended dose of NPK, code: fly ash + ½ NPKS
7. 5 t ha^{-1} of pressmud compost based on distillery effluent + half the recommended dose of NPKS, code: pressmud + ½ NPKS
8. 5 t ha^{-1} of dry *Jatropha* leaves + half the recommended dose of NPKS, code: Jatropha + ½ NPKS.

The fly ash + distillery effluent mixture (1:1) and the distillery effluent-based pressmud compost were obtained from Jubilant Organosys Ltd., Gajraula, Uttar Pradesh, India. The composition of the agro-based industrial wastes and the quantity of nutrients added with each are given in Tables 1 and 2.

Field and laboratory techniques

The organic materials were added to the soil after initial land preparation and two weeks before the sowing of rocket salad. After the addition of organic materials a good moisture content was maintained in the soil through irrigation. Rocket salad cv. RTM 314 was sown in the first week of November in both years at an inter-row spacing of 30 cm. Hand weeding and thinning were carried out 20 days after sowing. The experimental crops were irrigated with water from a borewell. The crop received two irrigations in addition to pre-sowing irrigation. The crop was

Table 1

Physicochemical properties of the 1: 1 mixture of fly ash and distillery effluent and of the distillery effluent-based pressmud compost used in the experiment

Parameters	1:1 mixture of fly ash and distillery effluent	Distillery effluent-based pressmud compost
pH	8.01	7.72
EC (dS m^{-1})	8.82	10.56
O.C. (%)	0.35	1.33
N (%)	0.31	2.15
P (%)	0.07	1.15
K (%)	1.57	2.60
Zn (mg kg^{-1})	5.32	2.58
Cu (mg kg^{-1})	16.62	91.1
Mn (mg kg^{-1})	17.36	347.8
Fe (mg kg^{-1})	42.48	58.57
Cd (mg kg^{-1})	0.02	–
Pb (mg kg^{-1})	2.10	–
Ni (mg kg^{-1})	1.75	–

Table 2

Quantities of organic material added, their NPK content and amount of NPK added/recycled

Organic material	Amount applied (t ha^{-1})	Concentration (%)			Amount of nutrients added/ recycled (kg ha^{-1} year^{-1})		
		N	P	K	N	P	K
1:1 mixture of fly ash and distillery effluent	5.0	0.31	0.07	1.57	15.5	3.5	78.5
Distillery effluent-based pressmud compost	5.0	2.15	1.15	2.60	107.5	57.5	130
Dry *Jatropha* leaves	5.0	1.33	0.25	1.27	66.5	12.5	63.5

harvested in the second week of March in both seasons. Half the N and the full dose of P, K and S was applied as per treatment before the sowing of the crop, whereas the remaining N was given at the time of the first irrigation. Data on growth and yield attributes were recorded for five randomly selected plants in each plot at harvest. The seed yield from each net plot area of 1.80 × 3.00 m was recorded and expressed as t ha^{-1}. Seed and stover samples of rocket salad were taken from five randomly selected plants in each season. The samples were oven dried at 70–80°C for 24 hours before analysis. The plant samples were ground in a cyclone mill, sieved and analysed for total nutrient content. The modified Kjeldahl method was used for total N content, while the seed and straw samples were digested in a diacid (HNO_3+$HClO_4$, 4:1) mixture for the estimation of P, K and S. P was estimated by the vandomolybdo-phosphoric acid yellow colour method, K by flame photometry and S by the turbidimetric method. Based on the N, P, K and S content in the seed and stover, the N, P, K and S uptake ha^{-1} was calculated by multiplying the dry weight with the respective nutrient content. Soil samples collected after the harvest of rocket salad each year were air-dried, screened through a 2 mm sieve, mixed, and analysed for pH, EC, organic carbon and available N, P and K by standard methods (Jackson, 1973). The oil content of *Eruca sativa* was determined by low resolution pulsed NMR spectrometry. The heavy metal contents in the fly ash + distillery effluent mixture and the distillery effluent-based pressmud compost were determined by atomic absorption spectrophotometry. After rocket salad, sorghum variety PC-6 was sown in the first week of April for fodder purposes and harvested by the first week of June. Green and dry fodder yield was recorded based on the net plot.

Statistical analysis

The experiments were conducted in a randomized block design for two consecutive years. Data on the growth, yield and yield attributes of the crops were recorded with three replications and pooled over years. All the experimental data were analysed at the 0.05% level of significance using SPSS.

Results and discussion

Growth and yield attributes

The treatments caused marked variations in the growth and yield components (Table 3). The treatments NPKS and pressmud + ½ NPKS gave statistically similar values of growth and yield components (plant height, primary and secondary branches plant^{-1}, siliqua plant^{-1} and oil content) except for the test weight. The values obtained for the growth and yield parameters in these two treatments were significantly superior to the application of pressmud, fly ash or Jatropha alone, to fly ash or Jatropha in combination with ½ NPKS and to the control. The integration of half the recommended dose of NPKS with the agro-industrial wastes caused a perceptible change in the plant height, primary and secondary branches plant^{-1}, siliqua plant^{-1}, test weight and oil content over the application of each organic material alone. These changes in the growth and yield components due to the treatments may be ascribed to variations in the amount of nutrients added through each material (Table 2), the extent of availability of the added nutrients, and changes expected in physicochemical properties due to the agro-industrial organic wastes. The organic sources of nutrients became more effective in improving the test weight and oil content when they were integrated with ½ NPKS, compared with their application alone. Maiti et al. (2003) reported similar changes in growth and yield attributes in Indian mustard, Zalawadia and Raman (1994) in sorghum and Azad et al. (1998) in wheat due to the integrated application of nutrients through organic and inorganic sources.

Seed yield and harvest index

The treatments NPKS and pressmud + ½ NPKS were statistically on par and resulted in significantly higher seed yield ha^{-1} and harvest index compared with the other treatments (Table 3). Pressmud + ½ NPKS increased the seed yield by 116.8, 80.0, 30.4, 63.6 and 24.1% over the control, fly ash alone, fly ash + ½ NPKS, Jatropha alone and Jatropha + ½ NPKS, respectively. Among of agro-industrial organic wastes applied alone, pressmud gave a marked increase in seed yield compared with fly ash and *Jatropha*, with increases of 37 and 27%, respectively. The variable beneficial effect of organic materials alone and in combination with NPKS on growth and yield attributes and thereby on the seed yield could be attributed to variations in the decomposition, mineralization and solubilizing effects on the fixed form of soil nutrients, causing variable supplies of available nutrients to the crop (Zalawadia and Raman, 1994; Singh et al., 2002). Of all the organic materials, pressmud compost showed better results because of the higher amount of nutrients added to the soil.

Table 3

Growth and yield attributes, seed yield and harvest index of rocket salad (*Eruca sativa*) and fodder yield of succeeding sorghum as influenced by integrated nutrient management in rocket salad (data pooled over two years)

Treatment	Rocket salad						Sorghum			
	Growth attributes			Yield attributes			SY	HI	GF	DF
	PH	PB	SB	SP	TW	OC				
1	85	4.6	4.5	76	3.15	31.3	0.83	19	10.5	2.64
2	115	6.7	8.5	155	3.57	34.7	1.69	24	12.2	3.1
3	90	5.0	5.3	92	3.25	31.4	1.0	20	11.0	2.78
4	102	5.3	6.3	133	3.56	33.3	1.37	21	12.3	3.20
5	95	4.7	5.2	107	3.33	31.6	1.10	21	11.4	2.84
6	109	5.6	5.9	121	3.40	32.2	1.38	23	12.0	2.96
7	122	5.9	8.7	162	3.73	35.2	1.80	25	13.4	3.32
8	113	5.6	6.0	138	3.48	33.2	1.45	23	12.3	3.08
CD $_{P=0.05}$	12	0.8	1.2	16	0.08	1.2	0.20	3	1.0	0.23

PH: Plant height (cm); PB: Primary branches plant^{-1}; SB: Secondary branches plant^{-1}; SP: Siliqua plant^{-1}; TW: Test-weight (g); OC: Oil content (%); SY: Seed yield (t ha^{-1}); HI: Harvest index (%); GF: Green fodder yield (t ha^{-1}); DF: Dry fodder yield (t ha^{-1}); 1. Control; 2: NPKS; 3: Fly ash; 4: Pressmud; 5: *Jatropha*; 6: Fly ash + ½ NPKS; 7: Pressmud + ½ NPKS; 8: *Jatropha* + ½ NPKS

Residual fertility after rocket salad

Changes in the soil-available NPK content were recorded due to the treatments given to rocket salad, compared to the initial soil fertility (Table 4). The highest content of available NPK was observed after the application of pressmud + ½ NPKS, which was markedly higher than the initial fertility status. Pressmud alone also resulted in a positive NPK balance. The recommended dose of NPKS led to a perceptible decrease in available N and K compared to the initial status, while in the case of P, the balance was positive. There was an improvement in the available NPK content due to different organic materials. This could be ascribed to the residual effect of agro-industrial organic wastes and their role in enhancing the availability of nutrients in the soil, as reported by Nanjappa et al. (2001).

Nutrient uptake by rocket salad

The total uptake of N, P, K and S in the seed + straw of rocket salad was significantly the highest for pressmud + ½ NPKS, followed by the recommended dose of NPKS (Table 4). The increase in total NPKS uptake due to pressmud + ½ NPKS was 8.4, 3.5, 9.5 and 6.0% over the recommended dose of NPKS and 136.1, 127.6, 103.5 and 52.8% over the control. The recommended dose of NPKS gave significantly higher NPKS uptake compared to the rest of the treatments, except for *Jatropha* + ½ NPKS, which was at par with NPKS for P and K uptake. For organic sources of nutrients applied alone, pressmud resulted in significantly the highest uptake of NPKS compared to fly ash and *Jatropha.*

The variation in nutrient uptake from the different treatments was the composite effect of variations in the quantity of added nutrients, changes in the physicochemical properties of the soil, seed and straw yield, and the nutrient content in the seed and straw.

Residual effect on the fodder yield of sorghum

On average, the fodder yield of succeeding sorghum was low due to the non-availability of irrigation water, but the residual effect of the treatments given to rocket salad was found to be significant (Table 3). Pressmud + ½ NPKS gave a significantly higher fodder yield than the other treatments. The recommended dose of NPKS, pressmud alone, fly ash + ½ NPKS and *Jatropha* + ½ NPKS gave similar green fodder yields, which were significantly superior to the control and to fly ash alone. These variations in fodder yield could be attributed to variations in the nutrients added through the various treatments and to their effects on the physicochemical properties of the soil. Similar results were reported by Singh et al. (2002), who found that organic sources play a key role in enhancing the efficient utilization of both native and added fertilizer nutrients.

The present study suggests that 5 t ha^{-1} distillery effluent-based pressmud compost can be used to replace half the recommended dose of NPKS, giving an improvement in the seed yield of rocket salad over the recommended dose of inorganic nutrients, while also resulting in a marked favourable effect on residual soil fertility and on the fodder yield of succeeding sorghum compared to the recommended dose of NPKS.

Table 4

Nutrient uptake by rocket salad (*Eruca sativa*) and soil fertility after its harvest, as influenced by organic and inorganic sources of nutrients (data pooled over two years)

Treatment	Nutrient uptake by seed and stover of rocket salad (kg ha^{-1})				Available nutrients after rocket salad harvest (kg ha^{-1})		
	N	P	K	S	N	P	K
1	37.6	7.6	64.9	12.5	190	11.5	195
2	81.9	16.7	120.6	25.0	210	13.6	198
3	48.1	10.8	80.7	14.1	195	11.6	215
4	65.1	14.1	103.4	22.4	222	15.8	222
5	50.6	10.8	89.1	16.2	210	13.3	218
6	67.0	13.8	110.6	20.1	210	13.9	218
7	88.8	17.3	132.1	26.5	235	16.6	226
8	70.5	14.6	114.8	21.2	218	14.6	223
CD $_{P=0.05}$	6.1	2.1	7.5	3.2			
INS					215	13.2	205

1. Control; 2: NPKS; 3: Fly ash; 4: Pressmud; 5: *Jatropha*; 6: Fly ash + ½ NPKS; 7: Pressmud + ½ NPKS; 8: *Jatropha* + ½ NPKS; INS: Initial nutrient status

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Short communication

IN VITRO PROPAGATION OF SOME SYRIAN VARIETIES OF OLIVE (*Olea europaea* L.)

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Five local olive varieties (*Olea europaea* L.), Dan, Sorani, Tadmury, Tiffahi and Zeiti, were collected from the field. Internode explants of each were micropropagated on modified MS and Rugini medium (olive medium).

The shoot growth and dry weight of the Dan, Sorani and Zeiti varieties increased significantly on modified MS. Similar results were observed for Tiffahi in Rugini medium. Modified MS and Rugini media had similar effects on the growth and dry weight of Tadmury.

Two concentrations (3 and 5 mg l^{-1}) of three auxins (IAA, IBA and NAA) were used for the *in vitro* rooting stage. A higher percentage of *in vitro* rooting was observed on medium containing 5 mg l^{-1} IBA.

Key words: MS, olive medium, tissue culture

Abbreviations: IAA: Indole-3-acetic acid; IBA: Indole-3-butyric acid; MS: Murashige and Skoog; NAA: α-naphthaleneacetic acid.

Introduction

Olive production occupies a prominent place in the economies of many Mediterranean countries (Rama and Pontikis, 1990). Olive is propagated worldwide by cutting, without any need for the addition of ionic solutions. In some Mediterranean countries grafting propagation is also applied (Lambardi and Rugini, 2003), but the number of plants propagated by this method remains small (Rama and Pontikis, 1990).

Although numerous experiments have been conducted with the manipulation of media and growth regulators (Cozza et al., 1997), the results are very much dependent on the genotype and the explant age (Rugini, 1984; Leva et al., 1992), so the success of olive micropropagation is still limited.

The use of *in vitro* culture, as a tool for the induction of genetic variation in olive clones and varieties through somaclonal variation and mutagenesis,

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depends on the possibility of establishing cultures of explants from field-grown plants, as well as on the definition of efficient methods of regeneration (Sofo and Calbi, 2001).

The present study was carried out to develop a protocol for the successful *in vitro* multiplication of some Syrian varieties, and to improve the multiplication rate and shoot quality.

Materials and methods

Woody plant cuttings of five local varieties, Dan, Sorani, Tadmury, Tiffahi and Zeiti, with 5–6 nodes were obtained from the field at the dormant bud stage.

All the cuttings were washed in water, and treated with 0.5 g l^{-1} Benomyl (fungicide; methyl-1-butylcarbamoyl-2-benzimidazole-carbamate 50%) for 30 min. Thereafter, they were washed in sterile water and dipped in a solution of 70% ethanol for 30 s and 2.5% commercial bleach for 3 min. After surface sterilization, they were washed three times with sterile water.

Shoots of 1–1.5 cm in length with one node were cultured in tubes containing 20 ml of the nutrient medium at the initiation and multiplication stage. Two nutrient media were used.

The first was Rugini medium (olive medium) (Rugini, 1984) containing 5 mg l^{-1} zeatine (Sofo and Calbi, 2001) and 30 g l^{-1} sucrose with added vitamins: 0.5 mg l^{-1} thiamine, 100 mg l^{-1} inosytol, 2 mg l^{-1} glycine, 5 mg l^{-1} nicotinic acid, 0.5 mg l^{-1} pyrodixine, 0.05 mg l^{-1} biotin and 0.5 mg l^{-1} folic acid. The pH of the medium was adjusted to 5.8 before adding 0.8% agar.

The second medium was MS (Murashige and Skoog, 1962), modified as follows: NH_4NO_3 was reduced to 825 mg l^{-1}, KNO_3 was increased to 2850 mg l^{-1}, $FeSO_47H_2O$ was increased to 41.77 mg l^{-1} and Na_2-EDTA was increased to 55.87 mg l^{-1}. The medium was supplemented with 3 mg l^{-1} zeatine and vitamins: 0.1 mg l^{-1} thiamine, 100 mg l^{-1} inosytol, 2 mg l^{-1} glycine, 0.5 mg l^{-1} nicotinic acid and 0.5 mg l^{-1} pyrodixine. The pH of the medium was adjusted to 5.8 before adding 0.8% agar.

The two media were autoclaved at 116°C for 25 min. All the cultures were incubated at 25°C with a 16-h photoperiod (140–150 µmol $m^{-2}s^{-1}$) under daylight fluorescent tubes (Phillips TLD 38/54).

Six subcultures of six weeks duration were done for each medium. At the end of the sixth subculture, half the micropropagated plants were dried at 70°C for 48 h and the shoot length, number of leaves and dry weight were measured.

The other half of the *in vitro* plants were transferred to rooting medium, consisting of Rugini (OM) or modified MS without zeatine. Both media were supplemented with 3 or 5 mg l^{-1} auxin (IAA, IBA or NAA).

The plants were kept under the conditions mentioned above for the 45-day multiplication stage. Thereafter, the percentage rooting was determined.

The experiment was arranged in a randomized complete block design (RCBD) with four replicates, each consisting of 16 plants. The experiment was repeated twice.

Statistical analyses were carried out using the StatView computer program (Abacus Concept, 1994) at the 95% confidence level ($P>0.05$). Analysis of variance (ANOVA) and the LSD test were used to determine significant differences between the means of the tested parameters.

Results and discussion

The medium has a major role in olive micropropagation (Cozza et al., 1997). Table 1 illustrates the effect of the medium on the growth of Syrian olive varieties *in vitro*.

The shoot lengths and dry weights of Dan, Sorani and Zeiti grown on modified MS medium increased significantly (*$P>0.05$*) as compared to those grown on Rugini medium (Table 1). Similar results were reported by Bartolini et al. (1989) for the cultivar Murino. In contrast, the data of shoot length, dry weight and leaf number were higher (*$P>0.05$*) for Tiffahi grown on Rugini medium than on modified MS, while both media promoted the shoot length and dry weight of Tadmury.

The number of leaves in Dan, Tadmury and Zeiti increased significantly ($P>0.05$) when the plants were grown in modified MS (Table 1). However, the two media had no significant effect on the number of leaves in Sorani.

These results confirmed the findings of Rugini (1984), who reported that zeatine is the best cytokinin for use in olive media. The results were in good agreement with those of Brhadda et al. (2003) on the cultivar Picholine marocaine.

Table 1

Effect of modified MS and Rugini medium on shoot length (cm), number of leaves, dry weight (mg) and rooting percentage of some Syrian olive varieties grown *in vitro*

		Varieties				
		Dan	Sorani	Tiffahi	Tadmury	Zeiti
Shoot length						
Modified MS		9.00a	7.28a	4.93b	8.38a	8.50a
Rugini		8.09	6.48b	8.08a	8.47a	8.47a
L.S.D.		0.51	0.43	0.36	0.4	0.36
Number of leaves						
Modified MS		9.71a	8.09a	8.96b	12.06a	11.46a
Rugini		8.25b	8.18a	12.00a	11.09b	9.06b
L.S.D.		0.53	0.85	0.69	0.93	0.8
Dry weight						
Modified MS		310a	240a	197b	248a	326a
Rugini		250b	208b	287a	252a	273b
L.S.D.		16	18	15	20	11
Rooting percentage						
Auxins	mg/l					
IBA	3	55b	49c	48c	47b	51b
	5	89a	85a	82a	76a	85a
IAA	3	37c	30e	33d	25d	32c
	5	47b	52b	56b	33c	52b
NAA	3	35c	27f	12f	20e	25d
	5	40c	33d	19e	25d	34c
L.S.D.		8.03	2.9	3.3	3.52	2.85

Values followed by the same letter are not significantly different at the 0.05 level (Fisher L.S.D.)

The total content of N and K was the main difference between the two media (Rugini and modified MS) used in the present experiment. N is reported to have a stimulating effect on organogenesis in olive (Brhadda et al., 2003), while Caboche (1987) found that the ratio of N/carbohydrates played an important role in the biosynthesis of growth regulators, and that NO_3 had a positive effect on plant morphogenesis. On the other hand, the increased K level in modified MS (2850 mg l^{-1} KNO_3) had a positive effect on the organogenesis and bud development of the plants, while the high content of K^+, Ca^{2+} and Mg^{2+} ions in the Rugini medium led to positive effects on plant organogenesis (David et al., 1978).

The percentage of rooting after 45 days was highest when a concentration of 5 mgl^{-1} IBA was used, compared to a concentration of 3 mgl^{-1} IBA and the other auxins (IAA and NAA). The highest percentage of rooting was observed for Dan (89%), whereas Tadmury had the lowest percentage (76%). These results were in agreement with those obtained by Rama and Pontikis (1990).

The positive effect of 5 mg l^{-1} IBA on the rooting percentage was demonstrated in the olive cultivar Kalamon by Rama and Pontikis (1990). This could be due to the rapid oxidation of IAA by plant tissues and to the faster plant metabolism caused by IBA than by IAA (Epstein and Ludwig-Muller, 1993).

The difference between IBA and NAA in the medium may be attributed to the fact that the latter is slowly oxidized by auxin-oxidase (Smulder et al., 1990). However, modified MS with IBA improved the rooting percentage and root quality, a result that was confirmed by Singh et al. (2004) for *in vitro* grapevine.

Overall, this study showed that the beneficial effect of modified MS could be observed for Dan, Sorani and Zeiti, while Rugini medium had a positive effect on Tiffahi. Tadmury could be grown on either medium. The application of 5 mg l^{-1} IBA was found to improve the rooting percentage of olive tissue cultures.

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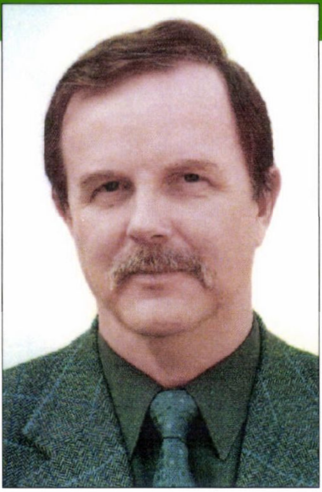

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ANTIOXIDANT ENZYMES IN GERMINATING WHEAT SEEDS AS AFFECTED BY DEHYDRATION STRESS, ABA AND HYDROGEN PEROXIDE

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The response of the antioxidant enzymes peroxidase [EC 1.11.1.11], superoxide dismutase (SOD) [EC 1.15.1.1] and catalase [EC 1.11.1.6] to dehydration stress caused by low and high temperature, salinity (0.2 M NaCl) and hyperosmoticum (0.5 M sucrose), as well as to exogenous ABA and H_2O_2, was examined in germinating wheat seeds. The data presented here confirm and complete previous results for other stages of wheat seedling development (Bakalova et al., 2004; 2007). Catalase was the most susceptible antioxidant enzyme under the chronic stress conditions applied. Its activity correlated closely to the decrease in the growth rate of wheat seedlings. Low temperature had the strongest effect of all the stress factors applied. There was a significant decrease in anionic peroxidase activity, accompanied by catalase inhibition, after low temperature treatment. An analysis of all the data obtained revealed that the treatments had mostly non-specific effects on gene expression, protein and enzyme profiles. Catalase and peroxidase activity were suppressed not only by low temperature, but by hyperosmoticum (0.5 M sucrose) as well. This result confirmed findings that a significant number of genes induced by one particular stress are also upregulated by other stresses (Kreps et al., 2002; Munns, 2002; Rabbani et al., 2003).

Key words: catalase, peroxidase, SOD, germination, dehydration stress, wheat

Introduction

Seedlings of many crop species from temperate regions are subject to different kinds of stress during germination and early growth stages under field conditions (Pinhero et al., 1997). Anderson et al. (1995) and Prasad (1996) showed that the stand establishment of maize is greatly affected by exposure of seedlings to low temperature during germination and early seedling growth. Chilling affects plants at all stages of their development. In particular, chilling injury is an important problem during seed germination and the post-germinative development of crops (Herner, 1990). The ability of seeds and young seedlings

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to cope with stress during early vegetative growth is vital for crop performance and production. Environmental stresses exert their effects directly or indirectly through the formation of reactive oxygen species (ROS) (Yu and Rengel, 1999). ROS are key players in the regulation of plant development, stress responses and programmed cell death (Gadjev et al., 2006; Gapper and Dolan, 2006; Halliwell, 2006). Depending on the quantity and type of ROS (hydrogen peroxide, superoxide and singlet oxygen) and its subcellular production site (plastidic, cytosolic, peroxisomal or apoplastic), different physiological, biochemical and molecular responses are stimulated (Vranova et al., 2002; Gadjev et al., 2006). ROS are produced in both unstressed and stressed cells (Alscher et al., 2002). The reactivation of the metabolism following seed imbibition may also provide an important source of ROS (Bailly, 2004; Wojtyla et al., 2006). The production and destruction of ROS under normal conditions is well balanced. However, under adverse environments such as extreme temperatures, salinity, drought or intense light, the formation of ROS is more rapid than scavenging and detoxifying, and this misbalance creates oxidative stress (Anderson et al., 1995; Baek and Skinner, 2003). It is now known that ROS are involved in seed development and the completion of seed germination, and the generation of ROS during seed desiccation, germination and ageing has been proven (Bailly, 2004; Wojtyla et al., 2006). ROS might also regulate changes in gene expression during seed development, dormancy and germination (Bewley and Black, 1994; Bailly, 2004). All changes in the resumption of the metabolism accompanying seed imbibition and germination contribute to ROS release and the activation of the antioxidant system (Wojtyla et al., 2006). Recently the dual role of ROS in plant and seed biology as both toxic molecules and signalling molecules, functioning in a wide range of responses to various stimuli, was unambiguously demonstrated (Bailly, 2004; Suzuki and Mittler, 2006). Therefore, antioxidant compounds and enzymes scavenging and detoxifying ROS are of particular importance for the survival of plants under stress (Foyer et al., 1994; Kreps et al., 2002; Foyer and Noctor, 2005; Mauro et al., 2005; Halliwell, 2006). Bailly (2004) proposed that cellular antioxidant mechanisms tightly control ROS concentrations rather than eliminating them completely. One of the mechanisms actively employed by plants to survive stress is the activation of the cell antioxidant system (Gechev et al., 2002). Various authors have shown that the amounts and activities of enzymes involved in ROS scavenging are altered by environmental stresses such as chilling (Prasad, 1996; Pinhero et al., 1997), salinity (Azavedo-Neto et al., 2005; Mandhania et al., 2006; Wahid et al., 2007), dehydration (Farrant et al., 2004) and drought (Srivalli et al., 2003). Numerous studies show that the isoenzymes of superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), and ascorbate peroxidase (APX) and their relative compositions change during exposure to stress, though in many cases the data obtained were very contradictory (Anderson et al., 1995; Prasad, 1996; Peyrano et al., 1997; Srivalli et al., 2003; Mauro et al., 2005).

The present study aimed to trace the early responses of antioxidant enzymes to dehydration stress in the seedling stage.

Materials and methods

Plant material

Field-grown wheat seeds (*Triticum aestivum*, cv. Sadovo 1) were used throughout the experiments. The seeds were germinated in filter paper rolls wetted with tap water, in darkness at optimal (24°C), low (10°C) or high (38°C) temperature or in the presence of 0.2 M NaCl, 0.5 M sucrose, 30 μM ABA or 10 mM H_2O_2 at optimal temperature. Pre-emergent conditions were simulated by growing the seedlings in the dark (Anderson et al., 1995). Sucrose, NaCl, high and low temperature exhibited certain similarities in the way they potentially created oxidative stress. All these treatments caused oxidative stress by inducing water deficit (dehydration). The endosperms and roots of wheat seedlings grown for 96 h were analysed. The treatments were repeated three times with different sets of seeds and replicate samples were taken from each treatment group. One set of these replicates was used for growth measurements and the other set for activity experiments on native gels.

Growth measurements

A batch of 100 seeds was used to determine the FW of endosperms and roots and the seedling length.

MDA content

The level of lipid peroxidation was measured in terms of malondialdehyde content, determined by the thiobarbituric acid (TBA) reaction according to the method of Heath and Packer (1968). A sample of 0.3 g was homogenized in 3 ml 5% TCA. The homogenate was centrifuged at 10 000 *g* for 20 min, after which 1.5 ml 20% TCA containing 0.5% TBA was added to 0.5 ml supernatant. The mixture was incubated at 95°C for 30 min and then quickly cooled in an ice-bath. The absorbance of the supernatant at 532 nm was read and the value of non-specific absorption at 600 nm was subtracted. The concentration of MDA was calculated using an extinction coefficient of 155 mM^{-1} cm^{-1}.

H_2O_2 content

The level of hydrogen peroxide in the endosperms and roots of the seedlings was measured by the iodometric method of Jessup et al. (1994) based on the oxidation of KI by H_2O_2 to I_2. A sample of 0.3 g was homogenized in 3 ml 5% TCA in an ice-bath. After centrifuging the homogenate at 12 000 *g* for 30 min, 0.5 ml 0.1 M K/Na phosphate buffer, pH 7.0, and 1.0 ml 1 M KI were added to 0.5 ml supernatant. The reaction was carried out in darkness at room temperature for 25 min. The absorbance of the supernatant at 390 nm was read. The concentration of H_2O_2 was calculated using an extinction coefficient of 25.6 mM^{-1} cm^{-1}.

Enzyme extraction

Cell-free extracts derived from seedlings subjected to various treatments were analysed for peroxidase, superoxide dismutase and catalase activities on non-denaturing gels. Freshly harvested endosperms and roots were ground in a mortar in 0.1 M tris-HCl buffer, pH 7.1. The material/buffer ratio was 1:5 for endosperms and 1:10 for roots. The homogenate was centrifuged at 12 000 *g* for 30 min at 4°C. The supernatant was used as a crude enzyme extract. Aliquots of enzyme extracts were mixed with equal volumes of 40% sucrose. All samples were stored at –20°C until enzyme analysis.

Total protein content determination

The protein content in the crude extracts was determined after TCA precipitation according to the method of Lowry et al. (1951) using BSA as a standard.

Native polyacrylamide gel electrophoresis

Native PAGE in 7.5% gel was carried out by the method of Davis (1964).

Enzyme visualization (after native PAGE)

Peroxidase isoenzymes were detected by incubating the gels (5 min for roots and 20 min for endosperms) in a reaction mixture containing 0.5 mM benzidine hydrochloride and 10 mM H_2O_2 in 0.05 M acetate buffer, pH 4.9, according to the procedure of Ornstein (1964).

Superoxide dismutase isoenzymes were detected on the gels by the method of Greneche et al. (1991). The gels were incubated for 30 min in the dark in a mixture containing 10 mg NBT, 75 mg Na_2-EDTA and 3 mg riboflavin dissolved in 100 ml tris-HCl buffer, pH 8.2. Subsequently the gels were illuminated for 15 min. The three SOD types were identified by performing activity staining in gels previously incubated for 20 min in 3 mM potassium cyanide or in 5 mM H_2O_2 (Donahue et al., 1997).

Catalase isoenzymes were stained as described by Woodbury et al. (1971). The gels were incubated in the dark for 20 min in 10 mM H_2O_2 dissolved in K/Na phosphate buffer, pH 7.0, followed by incubation in a mixture of 1% $K_3Fe(CN)_6$ and $FeCl_3$ for 15 min.

All the stained gels were fixed in a mixture containing H_2O: ethanol: acetic acid: glycerol (2:1:1:1). The stained isoenzyme patterns (cylindrical gels) were scanned densitometrically in the case of endosperm catalase. The differences and identity between individual isoenzymes were assessed by their number and the values of the relative electrophoretic mobility (Rm).

Statistics

All the determinations were performed in at least three replicates in 3–5 independent experiments. The significance of the differences was determined by Student's t-test, and P values of $R \leq 0.05$ were considered significant. The mean values ±SD are represented in the figures.

Results

Plant growth measurements were made as an indication of stress. Plant growth, in terms of root fresh weight and root length, was reduced in all seedlings subjected to stress factors (Fig. 1). There were no significant differences between the FWs of the endosperms. Significant changes between treatments were observed for root weight and length. Low temperature treatment caused the greatest growth retardation (90%), followed by sucrose (72%), high temperature (58%), ABA (57%) and NaCl (40%). The root length of seedlings exposed to low temperature was 98% lower, followed by sucrose (74%), ABA (71%), high temperature (53%) and NaCl (24%). The effect of H_2O_2 was weak for both parameters, causing a reduction of 5% in FW and 18% in root length. The soluble protein content of the seedlings was also affected by the stress factors applied (Fig. 2). A negative correlation between growth rate and protein content was obtained. The protein content in the roots was higher after treatment with low and high temperature or sucrose compared to the control roots, especially in the case of low temperature (Fig. 2B).

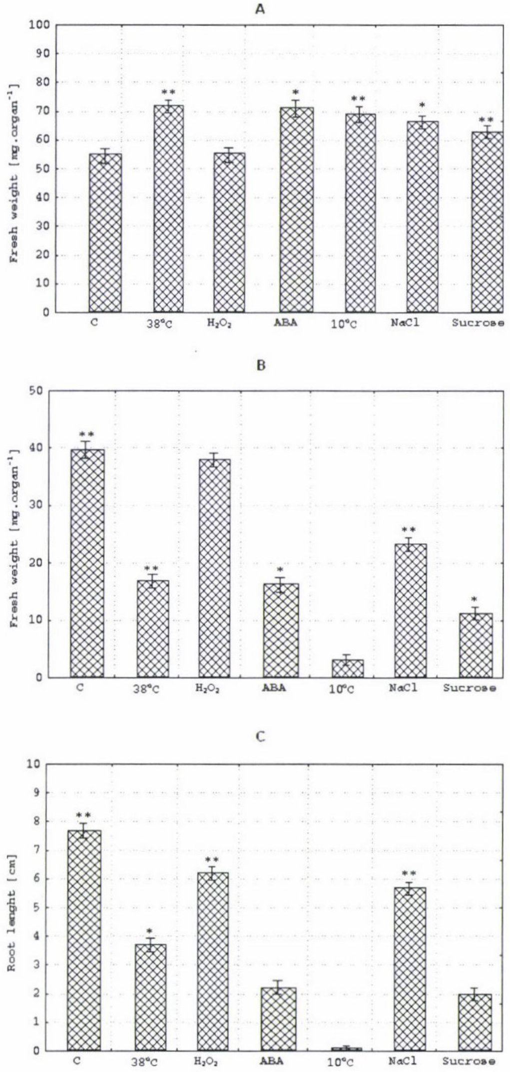

Fig. 1. Changes in the fresh weight of endosperms (A) and roots (B) and in the root length (C) of germinating wheat seeds. A hundred seeds were used for growth measurements. Data are expressed as mean ± SD of five independent experiments, $^{*}P<0.05$, $^{**}P<0.01$

The MDA content also changed depending on the stress factor applied (Fig. 3). A significant increase in the endosperm MDA content was found for sucrose (95%) and NaCl (84%), while only a slight rise in this parameter was detected for low temperature (32%). All the stress factors that affected root FW and length increased the MDA content in the roots as well.

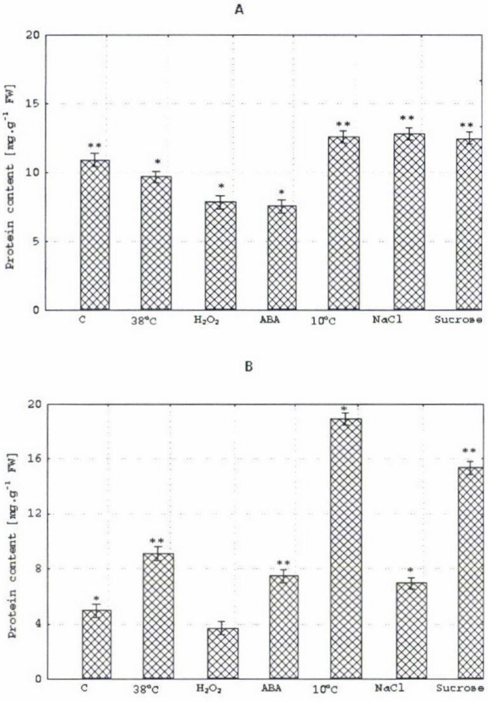

Fig. 2. Protein content in the endosperms (A) and roots (B) of wheat seedlings germinated under different conditions. Data are expressed as mean ± SD of three independent experiments, $^{*}P<0.05$, $^{**}P<0.01$

The stress factors were observed to havea slight effect on H_2O_2 content in the endosperms of the seedlings (Fig. 4A). The peroxide level in plants subjected to high temperature and ABA treatments was lower than in the control. In cold and sucrose treatment a slight H_2O_2 increase was observed in the endosperms. The highest H_2O_2 content was detected in the roots of the control seedlings. After all other treatments the level of H_2O_2 was lower than in the control roots (Fig. 4B).

Seven anionic isoperoxidases were detected in the endosperms of control seedlings (Fig. 5A). The lowest number of isoenzymes, as well as the lowest peroxidase activity, was revealed in endosperms exposed to low temperature treatment. An increase in the activity of isoenzymes N 5 and N 6 could be seen under the influence of high temperature and ABA. The slow migrating isoenzymes N 3 and N 4 disappeared in the endosperms after all the treatments. Twelve isoperoxidases were detected in the roots of control seedlings (Fig. 5B). Low temperature significantly decreased the activity of all the peroxidase isoenzymes (Fig. 5B). High temperature treatment increased the activity of isoenzymes N 8, N 11 and N 12 compared to the control roots. Treatment with ABA, NaCl and sucrose increased the activity of the moderately fast-migrating isoenzymes N 5 and N 7 (Fig. 5B).

Three SOD isoenzymes were stained in the endosperms of wheat seedlings (Fig. 6A), with isoenzyme N 4 being the most active. A slight decrease in its activity was observed at high temperature. In contrast, an enhancement in the activity of this isoenzyme was observed under the influence of low temperature and sucrose treatment. Five SOD isoenzymes were found in the roots of the seedlings after all treatments (Fig. 6B). Isoenzyme N 4 showed the highest enzyme activity, as also found in the endosperms. The lowest SOD activity was detected in roots treated at low temperature. All the other treatments had a positive effect on SOD activity. High temperature, ABA and sucrose significantly enhanced the activity of isoenzymes N 3, 4 and 5 (Fig. 6B), while H_2O_2 and NaCl increased the activity of isoenzyme N 4.

Treatment with the inhibitors H_2O_2 and KCN (Fig. 7) showed that one SOD band in the endosperms and three bands in the roots (Fig. 7A, B) were H_2O_2- and KCN-resistant, indicating that isoenzyme N 3 in the endosperm and isoenzymes N 1, 2 and 3 in the roots were MnSODs. Isoenzyme N 4 in the endosperms and isoenzymes N 4 and N 5 in the roots were FeSOD, since they were inhibited by H_2O_2 but not by KCN treatment. The fastest moving band, N 5, in the endosperm was a CuZn SOD isoform, because it was H_2O_2- and KCN-sensitive.

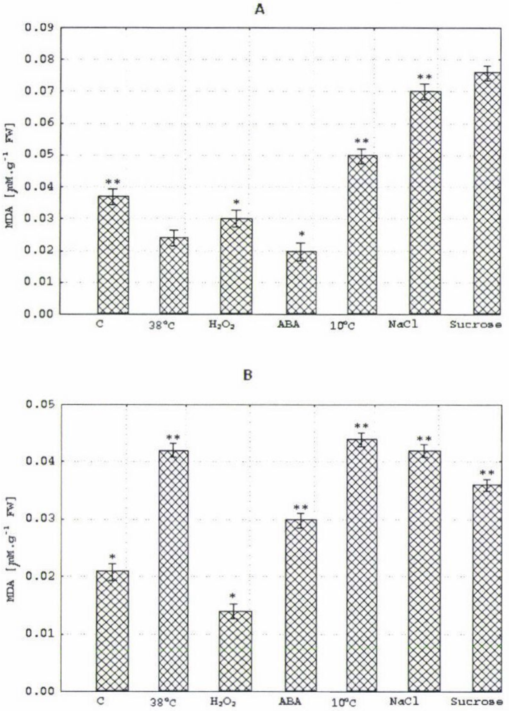

Fig. 3. MDA content in endosperms (A) and roots (B) of wheat seedlings germinated under different conditions. Data are expressed as mean ± SD of three independent experiments, $^{*}P<0.05$, $^{**}P<0.01$

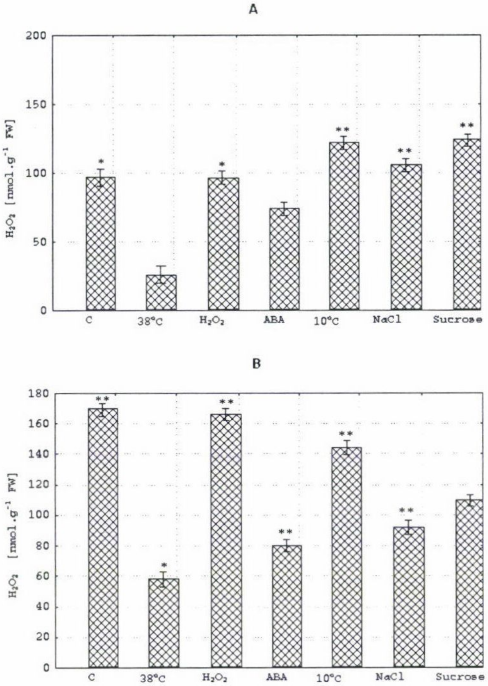

Fig. 4. H_2O_2 content in endosperms (A) and roots (B) of wheat seedlings germinated under different conditions. Data are expressed as mean ± SD of three independent experiments, $^{*}P<0.05$, $^{**}P<0.01$

Three catalase isoenzymes were revealed in the endosperm, the slowest moving being the most active (Fig. 8A). An increase in catalase activity was detected after ABA treatment. High temperature decreased catalase activity, while strong inhibition was detected in the endosperms after low temperature treatment. A similar electrophoretic profile was observed in the root samples. Low temperature significantly decreased the catalase activity in the roots (Fig. 8B).

Discussion

Results demonstrating growth retardation proved the stress effect of the factors applied (Fig. 1) and are in agreement with previous data for earlier stages of seedling development (24, 48, 72 h of germination) (Bakalova et al., 2004). At this stage of development (96 h) a partial recovery in the growth rate was observed in the case of high temperature, 0.2 M NaCl and especially in the case of H_2O_2 treatment. Yu and Rengel (1999) reported growth retardation in lupine plants treated with 100 mM NaCl for 2 days. Vranova et al. (2002) considered plant growth reduction via ROS participation to be part of an acclimatory mechanism. Under severe stress, a plant adapts its metabolism and alters its development (Bohnert et al., 1995).

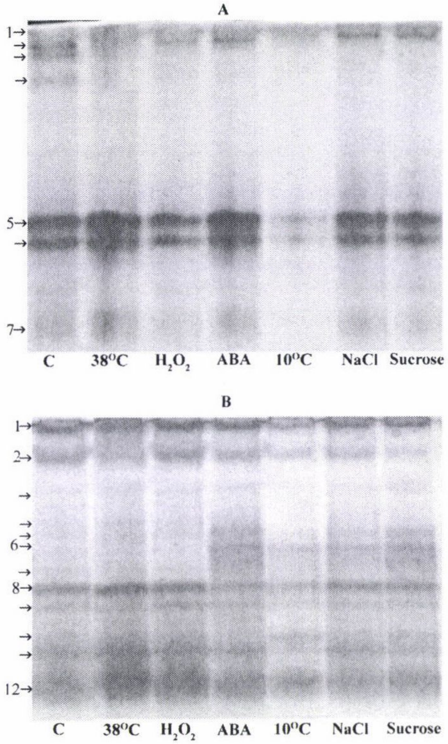

Fig. 5. Electrophoretic profiles of anionic peroxidase isolated from endosperms (A) and roots (B) of wheat seedlings. Anionic isoperoxidases were separated in 7.5% PAGE by the method of Davis (1964). Fifty micrograms of total protein was loaded per lane. Isoenzymes were visualized with benzidine as H-donor. Brown bands with peroxidase activity appeared after 10 min of incubation for roots and 30 min for endosperm isoperoxidases

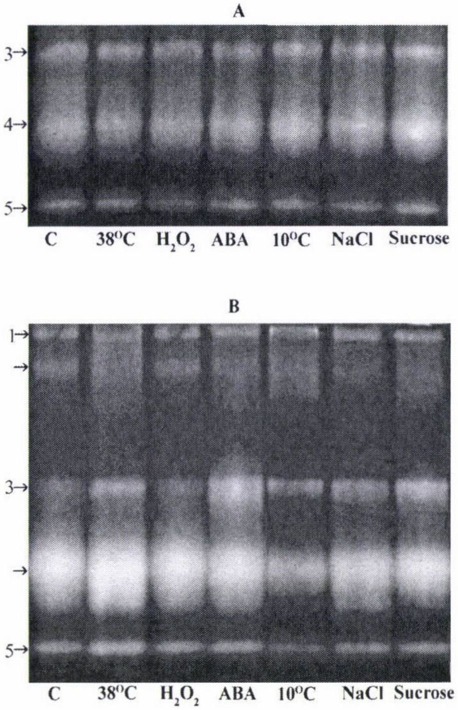

Fig. 6. Electrophoretic profiles of SOD isolated from endosperms (A) and roots (B) of wheat seedlings. SOD isoforms were separated in 7.5% PAGE by the method of Davis (1964). Fifty micrograms of total protein was loaded per lane. Isoenzymes were detected on plates by the staining procedure of Greneche et al. (1991) with NBT, EDTA and riboflavin. SOD isoforms were revealed as achromatic bands on a dark blue background after 3 min

The pronounced negative correlation between growth rate and protein content in the roots, established in a previous investigation, was confirmed (Bakalova et al., 2007).

A negative correlation was found between the growth rate and the extent of lipid peroxidation in the seedling roots. All the stress factors that decreased FW and root length increased the MDA content. The values obtained for MDA content resembled the results of Bailly et al. (2004) for sunflower seeds. The degree of lipid peroxidation was observed to be higher in the seedling roots than in the endosperms, probably because dividing and growing cells were more sensitive to ROS than the endosperms, where death cells dominated.

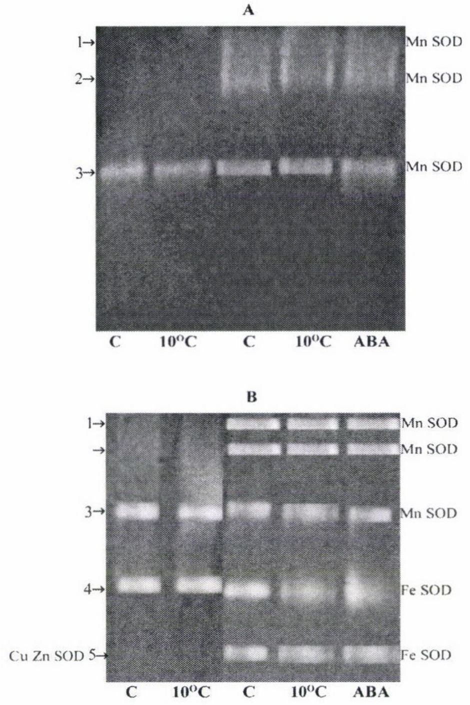

Fig. 7. Electrophoretic profiles of SOD isoenzymes isolated from endosperms (lanes 1, 2) and roots (lanes 3, 4 and 5) of wheat seedlings. Gels were pretreated with 5 mM H_2O_2 (A) or 3 mM KCN (B) for 20 min before activity staining. Fifty micrograms of total protein was loaded per lane

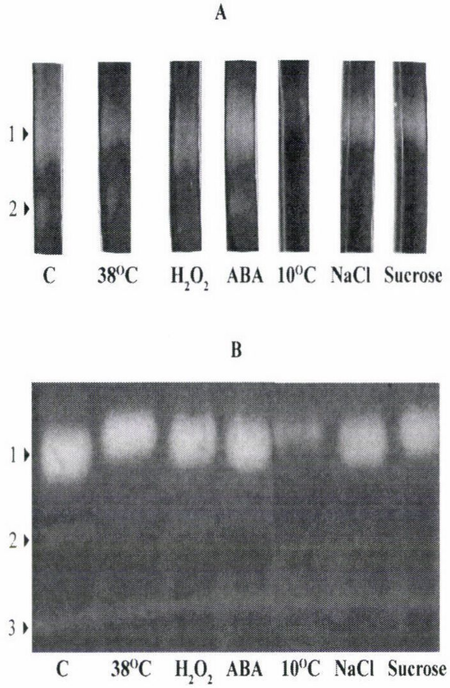

Fig. 8. Electrophoretic profiles of catalase isolated from endosperms (A) and roots (B) of wheat seedlings. Catalase isoenzymes were separated in 7.5% PAGE by the method of Davis (1964). 50 μg of total protein was loaded per lane for roots and a 100 μg per tube for endosperm using the staining procedure of Woodbury et al. (1971). The isoenzymes appeared as achromatic bands on a dark green background after incubation in 1% solution of $FeCl_3$ and $K_3Fe(CN)_3$ for 5 min (roots) and 10 min (endosperms).

Very high H_2O_2 levels were found in the endosperms (600 nmol g^{-1} FW) and embryos (100 nmol g^{-1} FW) of dry seeds in contrast to the data of Wojtyla et al. (2006) for the embryos and cotyledons of pea seeds, where H_2O_2 was not detectable. During seedling growth (96 h) the level of H_2O_2 significantly decreased in the endosperms, but in the roots an enhancement of 70% was observed due to the intensive reactivation of the metabolism in the cells of growing roots. In the endosperms, where the activity of the metabolism is lower due to the dominance of death cells, the H_2O_2 level declined. The data of Bailly et al. (2004) and Wojtyla et al. (2006), who showed that in cells with an intensive metabolism the H_2O_2 level was also high, support this hypothesis. Anderson et al. (1995) found no significant changes in the H_2O_2 level in the roots compared to shoots under the influence of cold treatment. According to these authors an adequate antioxidant system is already in place in the roots.

Low temperature significantly decreased peroxidase activity both in the endosperms and in the roots. These data are in agreement with the results of Oidaira et al. (2000) for rice seedlings and Anderson et al. (1995) for maize roots. The depression of peroxidase activity by low temperature contradicted the statement that an increase in peroxidase activity is a common response to various oxidative stress factors (Gaspar et al., 1985). The stimulating effect of high temperature, ABA, NaCl and sucrose treatment on the enzyme activity of moderately and fast-moving isoenzymes confirms the data of Srivalli et al. (2003) for rice peroxidase, while Peyrano et al. (1997) found no change in the overall peroxidase activity in the roots of salt-treated tomato plants. In the present study no induction of any new isoenzymes was observed in the profile of anionic peroxidase. Similar results were shown by Anderson et al. (1995). The effect of the stress factors applied was expressed as changes in the activity of individual existing isoenzymes.

SOD activity was affected by the stress factors applied in a specific manner. Low temperature decreased the activity of FeSOD isoenzyme N 4 and did not change the activity of MnSOD in the roots, in contrast to the results of Baek and Skinner (2003), who found a significant increase in MnSOD expression in winter wheat during cold acclimation. Anderson et al. (1995) found that root SOD activity did not undergo major changes during chilling. High temperature, ABA and sucrose significantly enhanced the activity of MnSOD isoenzyme N 3 and FeSOD isoenzymes N 4 and N 5 in the roots. Hydrogen peroxide and NaCl had a positive influence on the activity of FeSOD isoenzyme N 4. Similar differential control of SOD activity was reported by Yu and Rengel (1999) for lupine SOD. Results showing that the slowly migrating bands in wheat roots are MnSOD are in agreement with the results of Donahue et al. (1997) and Mauro et al. (2005) on the MnSOD isoenzymes in maize leaves. In contrast to these authors and the data of Wojtyla et al. (2006) no CuZnSOD isoenzymes were detected in wheat roots at this early stage of seedling growth in the present study. The other two fast-migrating isoenzymes stained were FeSOD, as proved by the inhibitory analysis data. By contrast, Wojtyla et al. (2006) reported that FeSOD isoenzymes were lacking in pea cotyledons.

One catalase isoenzyme was detected in the roots and endosperms of wheat seedlings, in agreement with the data of Wojtyla et al. (2006). Low temperature significantly decreased the catalase activity in the endosperms and roots of wheat seedlings (Fig. 8) in parallel with a decreased germination rate. According to Schafer and Feierabend (2000), catalase is known to be inactivated by various stress conditions that suppress protein synthesis, as has been shown for cold treatment. In rice grains, Tanida (1996) demonstrated that the germination rate at suboptimal temperature was positively correlated with CAT activity. Similar results were presented by Bailly et al. (1998) for sunflower seeds. Tanida (1996) suggested that the tolerance of rice cultivars to chilling injury was closely linked to the cold stability of catalase and ascorbate peroxidase. Anderson et al. (1995) considered that one effect of chilling stress could be the oxidative inactivation of catalase. The present results confirm those of Baek and Skinner (2003), who reported a prolonged decrease in catalase expression under the influence of low temperature. In the present work an increase in catalase activity was observed under the influence of ABA treatment. A similar effect of ABA on catalase (*Cat 1*) in developing maize grains was presented by Guan and Scandalios (1998), who considered that changes in catalase transcripts and isoenzyme activities in response to ABA might be caused, in part, by the altered metabolic activity of the cells, leading to changes in ROS levels.

The data presented here confirm and complete the results previously obtained for other stages in the development of wheat seedlings (Bakalova et al., 2004; 2007). Catalase was the most susceptible antioxidant enzyme under the chronic stress conditions applied. Its activity correlated closely to the decrease in the growth rate of wheat seedlings. Low temperature had the strongest effect of all the stress factors applied. Comparative analysis of the changes in antioxidant enzyme profiles showed that, in parallel to catalase inhibition by low temperature, there was a significant decrease in the activity of the anionic peroxidases (Fig. 5). Further investigations using the Real Time PCR technique showed that the expression of genes encoding peroxidase and catalase enzymes was suppressed (Bakalova et al., unpublished data). Similar results were presented by Janda et al. (2003). The CAT gene expression profile declined very strongly in accordance with the significant reduction in catalase activity in cold-acclimated wheat leaves. The present results confirm those of Bohnert et al. (1995), who claimed that the alteration of gene expression is always involved in preparing plants for survival under stress.

As a common feature of the stress response of peroxidase, superoxide dismutase and catalase in the present study, it was revealed that the changes in enzyme activity were brought about by existing isoenzymes. This contradicts the opinion of Rao et al. (1996), who considered that the synthesis of new isoenzymes of antioxidant enzymes might be more beneficial for the ROS metabolism.

The interpretation of plant responses to stress is exceedingly complex, because the mechanisms involved differ depending on species, tissues, stage of development, physiological status of the plant, and the degree and duration of the stress (Baek and Skinner, 2003). Anderson et al. (1995) reported that the effect of chilling on antioxidant enzymes differed depending on the tissue; the present data showed that this dependence also existed for the other types of stress factors. The roots were visibly more susceptible to stress damage than the endosperms.

Some authors reported that the limitation of one component in the antioxidant system could be compensated through the up-regulation of other elements in the stress defence system (Willekens et al., 1997), but the present results did not confirm this. For example, low temperature decreased the activity of both the catalase isoforms and the anionic and cationic isoperoxidases without causing any change in SOD activity.

An analysis of all the data obtained confirms that the exogenous factors applied mostly had non-specific effects on gene expression and on protein and enzyme profiles. Catalase and peroxidase activity were found to be suppressed not only by low temperature treatment, but by hyperosmoticum (0.5 M sucrose) as well. These results confirmed the suggestion that a significant proportion of the genes induced by one particular stress could be induced by other stresses as well (Kreps et al., 2002; Munns, 2002; Rabbani et al., 2003).

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EFFECTS OF LONG-TERM SALICYLIC ACID PRE-TREATMENT ON TOMATO (*Lycopersicon esculentum* MILL. L.) SALT STRESS TOLERANCE: CHANGES IN GLUTATHIONE S-TRANSFERASE ACTIVITIES AND ANTHOCYANIN CONTENTS

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The aim of the present study was to investigate the effect of salicylic acid (SA) pre-treatment on the salt stress acclimation of tomato plants (*Lycopersicon esculentum* Mill. L. cv. Rio Fuego). The antioxidant defence and detoxifying capacity of the tissues were analysed by measuring the accumulation of soluble, non-enzymatic antioxidants (anthocyanins) and the activities of glutathione S-transferases (GSTs) at low (10^{-7} M) and high (10^{-4} M) SA concentrations in plants exposed to 100 mM NaCl. GSTs are a diverse group of enzymes that catalyse the detoxification of xenobiotics and other toxic organic compounds, and anthocyanins are among the few endogenous substrates that bind to GSTs and are sequestered to the vacuole. It was found that 10^{-4} M SA pre-treatment improved the acclimation of tomato to high salinity. SA pre-treatments increased the accumulation of anthocyanins both in the presence and absence of 100 mM NaCl. The extractable GST activity of tissues increased under salt stress in young leaves and roots of the control and in plants pre-treated with 10^{-4} M SA, while the extractable GST activity in these organs was reduced by 10^{-7} M SA. It is suggested that elevated GST activity is a prerequisite for successful acclimation to high salinity in tomato plants pre-treated with SA, but it may also be a symptom of tissue senescence.

Key words: anthocyanin contents, glutathione S-transferase activity, *Lycopersicon esculentum* Mill. L., salicylic acid, salt stress

Introduction

Salinity is one of the most frequent and most deleterious stress factors in arid and semi-arid regions, which may severely limit crop production. The most harmful effects of salinity are nutritional imbalance in plant tissues due to enhanced Na^+ concentration and the low osmotic potential of the soil solution. Salt stress induces changes in ion transport (uptake, extrusion and sequestration of ions) and the metabolism (synthesis of compatible solutes) and results in morphological and developmental alterations in plants. The primary osmotic

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stress may result in secondary effects. The inhibition of photosynthetic performance and the generation of reactive oxygen species (ROS) also play a role in plant responses to salt stress.

The most important processes of salt stress tolerance are the maintenance of ion homeostasis, the detoxification of harmful compounds and the recovery of growth. Tomato (*Lycopersicon esculentum* Mill. L.) is a glycophyte that has been considered as a moderately salt-sensitive plant. Salt stress imposes both an ionic and an osmotic stress on plant tissues. The cytosolic and organellar machineries of halophytes and glycophytes are equally sensitive to Na^+ and Cl^- ions; consequently the vacuolar compartmentalization of these ions is essential (Greenway and Osmond, 1972; Flowers et al., 1986; Munns, 1993; Serrano, 1996; Zhu, 2003). Processes important for ion homeostasis include the cellular uptake, sequestration, export and long-distance transport of Na^+.

Compartmentalization not only mitigates the toxic accumulation of ions in the cytoplasm, but is also an important mode of osmotic adjustment in saline environments, which is necessary for growth and development (Hasegawa et al., 2000).

The phenolic compound salicylic acid (SA) has been identified as a key signalling component in plant responses to biotic stress factors. Moreover, the application of exogenous SA enhanced the resistance of tomato plants to drought (Senaratna et al., 2000) and salt stress (Tari et al., 2002), but the results were contradictory in the case of other species and depended on the developmental phase of the plants (Borsani et al., 2001) or on the experimental conditions (Németh et al., 2002).

Salt stress also generates secondary effects, particularly oxidative stress, which is caused by the excess of reactive oxygen species, e.g. hydrogen peroxide, hydroxyl radicals and superoxide anions. The elimination of ROS is mainly achieved by antioxidant compounds such as ascorbic acid, glutathione and carotenoids, and by ROS scavenging enzymes, e.g. superoxide dismutase (SOD, EC 1.15.1.1), ascorbate peroxidase (APX, EC 1.11.1.11) and guaiacol peroxidase (POD, EC 1.11.1.7) (Agarwal et al., 2005; Taşgın et al., 2006). The ROS generated in cells exposed to unfavourable external conditions may destroy the normal cellular function and metabolism. It has been observed that water-soluble antioxidants, anthocyanins, accumulate during salt stress in tomato seedlings (Eryilmaz, 2006), and the expression of the enzymes involved in their biosynthesis (leucoanthocyanidin dioxygenase-like protein, putative anthocyanidin synthase and UDP rhamnose-anthocyanidin-3-glucoside rhamnosyltransferase-like protein) also increased after salt stress in *Arabidopsis* (Col-0 *gl1*) (Gong et al., 2001).

SA specifically binds to certain catalase isoenzymes and inhibits the enzyme activity (Chen et al., 1993; Horváth et al., 2002), which may lead to an increase in the H_2O_2 content of tissues (Rao et al., 1997). Mittova et al. (2002) determined the role of various antioxidants in the salt tolerance of tomato species and found that, as compared to cultivated tomato (*Lycopersicon esculentum* Mill. L.), the higher salt tolerance of wild tomato (*Lycopersicon pennellii*) was correlated with increased activities of SOD, APX and POD.

To explore the protective role of SA in the antioxidant defence responses induced by salt stress, investigations were made on changes in the activity of an antioxidant and detoxifying enzyme, glutathione S-transferase (GST, EC 2.5.1.18). GSTs provide protection against environmental stress and disease mainly by detoxifying reactive and/or toxic products generated by oxidative stress. They catalyse the transfer of glutathione to a co-substrate of the enzyme containing a reactive electrophylic centre. The anthocyanins are non-enzymatic substrates of GSTs, and after binding with enzymes they are transported to the vacuole (Mueller et al., 2000). GSTs are active in herbicide detoxification and are also important factors in the chilling and salt tolerance of transgenic tobacco (Basantani and Srivastava, 2007; Roxas et al., 1997). They may also act as regulators of apoptosis (Kampranis et al., 2000).

Some GST genes, such as *GST6* in *Arabidopsis,* have been observed to be regulated by SA or H_2O_2. The *GST6* promoter contains a 20-bp octopine synthase (*ocs*) element, which is a binding site for bZIP proteins but is also responsive to H_2O_2. The *GST6* promoter is thus induced in roots by SA and H_2O_2 (Chen and Singh, 1999).

The present work investigated changes in the total activity of GSTs and the accumulation of non-enzymatic antioxidants (anthocyanins) in tomato plants pre-treated with SA under salt stress. Some of the isoenzymes of GSTs and the synthesis of anthocyanins may also be induced by H_2O_2 (Lois, 1994), so the aim was to discover whether different concentrations of SA affected the extractable activity of GSTs and modified the accumulation of anthocyanins in tomato plants under salt stress.

Materials and methods

Plant material and salinity treatments

Tomato (*Lycopersicon esculentum* Mill. L. cv. Rio Fuego) plants were grown hydroponically in the greenhouse under 180 µmol $m^{-2}s^{-1}$ light intensity and a 12/12 h day/night photoperiod. The temperature was maintained at 26°C and the relative air humidity was 55–60%. The seedlings were transferred to perlite and grown until 2 weeks of age. From the third week they were grown in nutrient solution. The nutrient solution contained the following chemicals: 2 mM $Ca(NO_3)_2$, 1 mM $MgSO_4$, 0.5 mM KCl, 0.5 mM KH_2PO_4, 0.5 mM Na_2HPO_4, 10^{-6} M $MnSO_4$, $5 \cdot 10^{-7}$ M $ZnSO_4$, 10^{-7} M $CuSO_4$, 10^{-7} M $(NH_4)_6Mo_7O_{24}$, 10^{-5} M H_3BO_4 and $2 \cdot 10^{-5}$ M Fe-EDTA (pH 5.8).

The plants were treated with 10^{-4} M or 10^{-7} M SA for three weeks. The medium was changed every 3 days. In the 6th week salt stress was induced by adding 100 mM NaCl to the hydroponic culture. Plants without NaCl treatment served as a control. Measurements were made 7 days after the salinity treatment on the second fully expanded young leaf, on lower older leaves and on roots, with three replicates.

Anthocyanin determination

The anthocyanins were determined as described by Lichtenthaler (1987) with the modification of Sims and Gamon (2002). Optical density was measured with a KONTRON Double-Beam spectrophotometer at 534, 643 and 661 nm. Pigment contents were calculated by means of the following formula:

Anthocyanin (μmol ml^{-1}) = $0.0821*A_{534}–0.00687*A_{643}–0.002423*A_{661}$

Lipid peroxidation determination

Malondialdehyde (MDA), routinely used as an indicator of lipid peroxidation, was extracted with 5% (w/v) trichloroacetic acid and determined according to Heath and Parker (1968).

Enzyme activity of glutathione S-transferase (GST, EC 2.5.1.18)

Enzyme activity was determined as a function of time for 1 week after 100 mM NaCl exposure. One g of plant tissue was homogenized on ice in 4 ml extraction buffer [50 mM phosphate buffer, pH 7.0, containing 1 mM EDTA, 1 mM phenylmethylsulphonyl fluoride (PMSF) and 1% polyvinyl-polypirrolidone (PVPP)]. The homogenate was filtered through two layers of cheese-cloth and centrifuged for 25 min at 15 000 *g* at 4°C. The supernatant was used for enzyme activity assay. The homogenization was repeated two or three times, and GST activity was determined spectrophotometrically by using an artificial substrate, 1-chloro-2,4-dinitrobenzene (CDNB), as described by Habig et al. (1974). Reactions were started by the addition of CDNB, and the increase in A_{340} was determined. One EU is the amount of enzyme that produces 1 μmol conjugated product in 1 min, $\varepsilon_{340}=9.6\ mM^{-1}cm^{-1}$. The protein contents of the extracts were determined by the method of Bradford (1976). The means ± SD were calculated from the data of at least three measurements. The data were analysed using Student's t-test at the 0.05 (*), 0.01 (**) or 0.001 (***) probability levels. In some cases a Duncan's multiple range test was performed and probability was assumed as $P \leq 0.05$.

Results

The protection afforded by SA against abiotic stressors is explained by the activation of enzymatic detoxifying and antioxidative processes. The present work investigated the effects of pre-treatments with two salicylic acid concentrations (10^{-7} M and 10^{-4} M SA) on the subsequent salt stress response of tomato plants exposed to 100 mM NaCl. The non-enzymatic (anthocyanins) and enzymatic (GSTs) antioxidant defence responses initiated by an excess of SA and by SA-induced H_2O_2 during salt stress were determined as a function of the SA concentrations used in the pre-treatment period.

It was found that SA enhanced the anthocyanin contents both in pre-treated controls and in pre-treated plants exposed to high salinity (Fig. 1).

The initial extractable activities of GSTs were different in various plant tissues. Since the promoter of GST genes may contain elements responsive to oxidative stress, these differences in basal activities may be explained by the different contents of ROS, especially H_2O_2, in the tissues (unpublished results). Moreover, the activities exhibited transient changes both in control and SA pre-treated plants after refreshing the nutrient solution on days 0 and 3.

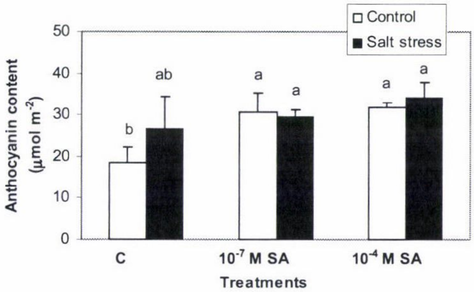


Fig. 1. Effect of three-week pre-treatments with 10^{-7} or 10^{-4} M salicylic acid on the accumulation of anthocyanins in tomato plants exposed to 100 mM NaCl for 1 week. Means±SD. Means denoted by different letters are significantly different at $P \leq 0.05$ as determined by Duncan's multiple range test

Salt stress decreased the activity of GSTs both in the young leaves (Fig. 2) and in the roots (Fig. 3) of tomatoes pre-treated with 10^{-7} M SA. By contrast, in control plants and in the case of pre-treatment with 10^{-4} M SA the activity of the enzyme either did not change significantly or increased in the young leaf and root tissues after exposure to 100 mM NaCl. This suggests that SA-induced GST activity may contribute to the successful acclimation of tomato plants to high salinity.

The concentrations of lipid peroxidation products, such as malondialdehyde, were also significantly reduced under salt stress in pre-treated plants (Fig. 4).

Discussion

Substances such as MDA, which react with thiobarbituric acid and act as markers of lipid peroxidation, decreased significantly in SA pre-treated plants under salt stress, suggesting the successful priming of antioxidant processes by SA. These results suggest that membrane damage was prevented to a considerable extent by the SA pre-treatments.

Reduced lipid peroxidation is also a resistance marker during salt stress in cultivated tomato genotypes (Juan et al., 2005). Similar results were also obtained in the wild tomato species *Lycopersicon pennellii* at high salinity (Shalata et al., 2001).

The anthocyanins are a group of water-soluble flavonoids that impart pink to purple colours in leaves and other organs. The occurrence of anthocyanin was evident in the epidermal cells of the stem of the Rio Fuego cultivar. These compounds are not only effective scavengers of ROS, but may upregulate *pi*-type GSTs in human retinal pigment epithelial cell cultures (Milbury et al., 2007) or *in vitro* may significantly inhibit a GST isoenzyme purified from oat seedlings (Zachariah et al., 2000). Thus, anthocyanins can both increase and decrease the activities of GSTs and their accumulation contributes to the ROS scavenging capacity of the tissues.

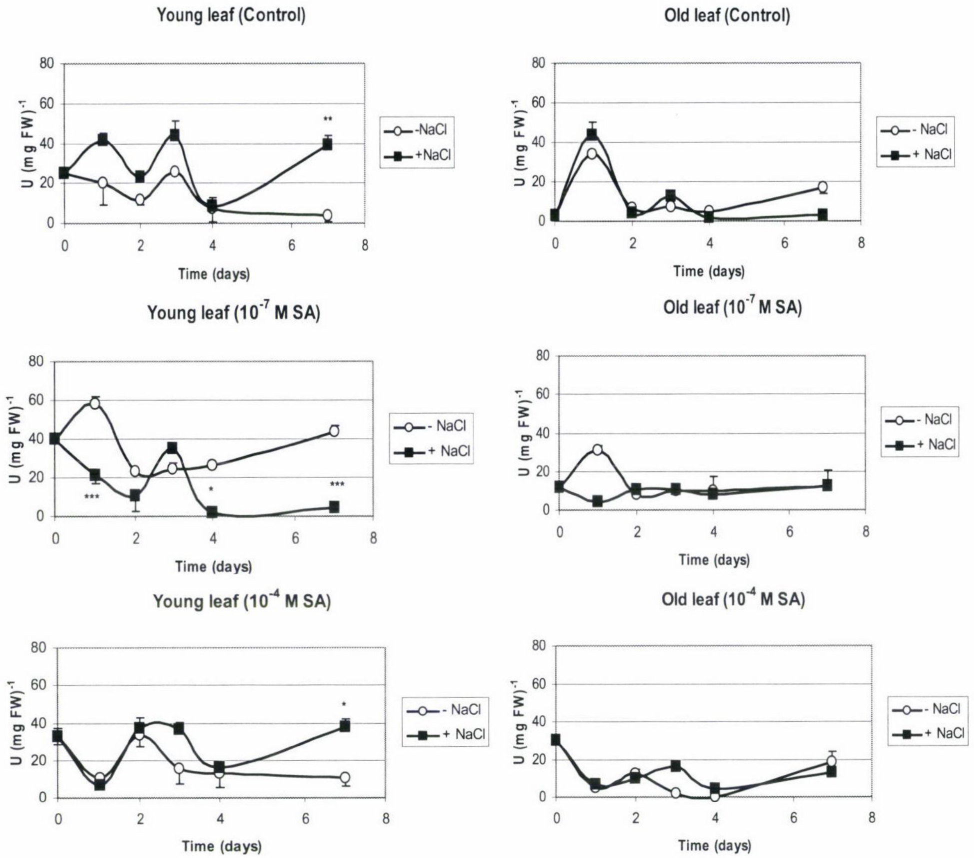
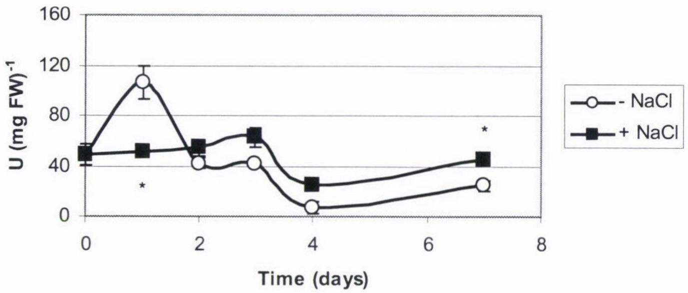


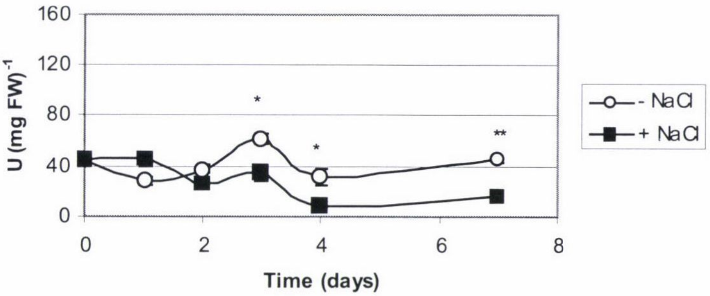
Fig. 2. Glutathione S-transferase (GST) activities in the leaves of tomato plants exposed to 100 mM NaCl for 1 week after three-week pre-treatment with 10^{-7} or 10^{-4} M salicylic acid. Means±SD, n=3. Means denoted by *, ** or *** are significantly different from the corresponding control at the P≤0.05, 0.01 or 0.001 levels, respectively

The GST superfamily in plants can be subdivided into eight classes (Basantani and Srivastava, 2007). *Phi-* and *tau*-class GSTs, which are specific to plants, may function as glutathione peroxidases and alleviate oxidative stress. They may also bind flavonoids (Mueller et al., 2000). The expression of some *phi*-class GSTs increased upon exposure to caffeic acid, 7,4-dihydroxyflavone and naringenin in wheat. Two of them proved to be sensitive to inhibition by flavonoids (Cummins et al., 2003). Thus, the expression of various types of GSTs may be enhanced by SA, SA-induced H_2O_2 (Chen and Singh, 1999) or flavonoids (Cummins et al., 2003), but the expressed protein activity may also be inhibited by these latter compounds. Thus, the effect of SA on extractable GST activity is a complex process. Salt stress alone increased the GST activity in the roots and young leaves. Acclimation of these plants to 100 mM NaCl failed during this period of time.

Root (Control)



Root (10^{-7} M SA)



Root (10^{-4} M SA)

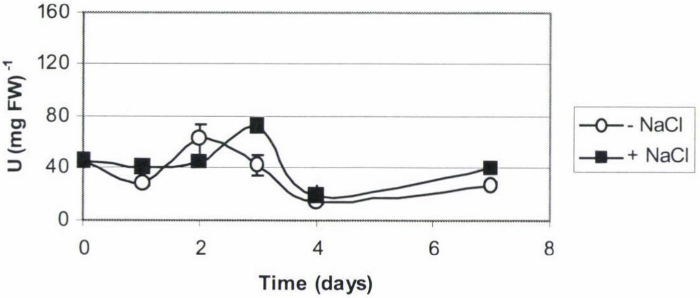


Fig. 3. Glutathione S-transferase (GST) activities in the roots of tomato plants exposed to 100 mM NaCl for 1 week after three-week pre-treatment with 10^{-7} or 10^{-4} M salicylic acid. Means±SD, n=3. Means denoted by * or ** are significantly different from the corresponding control at the P≤0.05 or 0.01 levels, respectively

Similar changes in GST activity may be observed after 10^{-4} M SA pre-treatment, but the acclimatization process to 100 mM NaCl proved to be successful. In plants pre-treated with 10^{-7} M SA, the enzyme activity either did not change significantly or decreased in the young leaves or roots. Since both low and high concentrations of SA increased the level of H_2O_2 in the tissues (unpublished results) and the non-enzymatic antioxidant content (e.g. anthocyanins) of the shoots during salt stress, it seems likely that the expression, protein level and enzyme activities of different GST isoenzymes are regulated in a complex way at the two concentrations of SA. Thus, high total GST activities may be a symptom of the onset of the senescence process in the case of unsuccessful acclimation or may be a prerequisite for adaptation to high salinity.

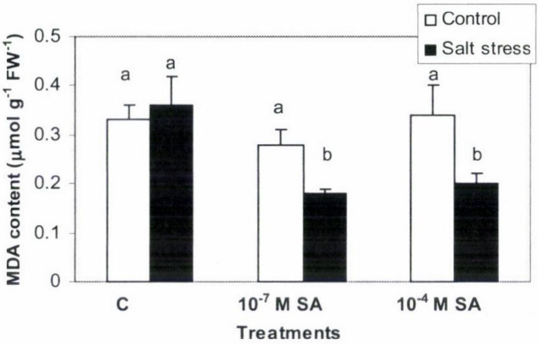


Fig. 4. Malondialdehyde (MDA) content (μmol g^{-1} fresh weight) in the SA-pre-treated leaves after 1 week of treatment with 100 mM NaCl. Means±SD. Means denoted by different letters are significantly different at $P \leq 0.05$ as determined by Duncan's multiple range test

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CHARACTERIZATION OF SALINITY TOLERANCE IN RICE (*Oryza sativa* L.) GENOTYPES AT THE GERMINATION AND SEEDLING STAGES

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The present work was conducted to study the genotypic variability of rice genotypes at the germination and seedling stages at different levels of salinity (0 M, 0.15 M, 0.2 M and 0.25 M NaCl). The results showed that increasing salinity decreased germination and seedling growth. Significant genotypic variability exists in the germination and seedling stages in response to different NaCl concentrations. Most of the genotypes showed more than 90% germination in the control, indicating good seed vigour. Two genotypes, VBR 638 (93%) and VBR 644 (84%), were selected as being tolerant to salinity at 0.2 M NaCl at the germination stage. Therefore, these could be used as source materials for genetic improvement for salinity tolerance at the germination stage. A considerable amount of genotypic variability was also found under control and saline conditions at the seedling stage with respect to the variables shoot height, root length, shoot and root dry weight. The high heritability observed for these variables offers good scope for genetic improvement for salinity tolerance both at the germination and seedling stages. The genotypes VBR 616, VBR 628, VBR 645, VBR 640, VBR 611, VBR 620, VBR 612, VBR 618, VBR 644, VBR 629, VBR 625 and VBR 630 were selected as being tolerant to salinity at the seedling stage.

Key words: *Oryza sativa*, rice, salinity tolerance

Introduction

Salinity affects crop production in rice-growing areas worldwide under a wide variety of growing conditions (Funakawa et al., 2000; Kotb et al., 2000; Wilson et al., 2000). Salt concentration causes osmotic stress, delaying water penetration into the cells, leading to dehydration of the protoplasm and thereby affecting seed germination and seedling growth and inhibiting enzyme activities, while also suppressing respiration and the phosphorylation process.

Salinity affects crop growth in rice, a salt-sensitive crop (Shannon et al., 1998) at different growth stages. Rice is more tolerant at germination than at other stages (Narale et al., 1969; Khan et al., 1997), but salinity nevertheless affects both germination and seedling growth. The inhibition of germination at high salinity levels might be due to osmotic stress (Narale et al., 1969; Heenan et al., 1988). Rice seedlings were highly sensitive to salinity (Pearson and Bernstein, 1959; Kaddah, 1963; Yeo and Flowers, 1986; Lutts et al., 1995). Salinity causes a significant reduction in seedling establishment, leading to losses in yield components and yield (Sajjad, 1984; Heenen et al. 1988; Khatun et al., 1995; Kato and Takeda, 1996; Scardaci et al., 1996; Shannon et al., 1998; Zeng and Shannon, 2000). It has been demonstrated by Azaizeh et al. (1992) that NaCl has adverse effects on water transport in root cells, but supplemental Ca^{2+} could compensate for these effects. This was also supported by Alam et al. (2005).

Materials and methods

The present study was conducted at the Seed Physiology Laboratory of Vibha Agrotech Ltd. (VAL), Hyderabad, India (Aug, 2005) using 29 rice genotypes, developed by VAL, having wide phenotypic variability.

Salinity tolerance of rice at the germination stage

Twenty-seven rice genotypes were used to evaluate their performance at the germination stage in Petri dishes under control conditions (T1 – distilled water) and under saline conditions (T2 – 0.15 M NaCl, T3 – 0.20 M NaCl and T4 – 0.25 M NaCl). Fifty seeds were used for each Petri dish. All the treatments were replicated twice. Germination was counted on the 7th day after sowing.

Variability, heritability and character association of rice genotypes at the seedling stage

Twenty-nine rice genotypes (including the 27 genotypes used in the germination test) were grown in plastic pots (200 mm) using sterilised sand at room temperature and light for 20 days. Twenty-five seeds were sown in each pot under control conditions (T1: distilled water + Knopp's nutrient solution) and at two salinity levels (T2: 0.15 M NaCl + Knopp's nutrient solution; T3: 0.20 M NaCl + Knopp's nutrient solution). Each treatment was replicated three times for all the genotypes. Observations were made on 20 randomly selected 20-day-old seedlings. Data were recorded for 1000-grain weight (which remained the same for all three growing conditions), shoot length (cm), root length (cm), shoot dry weight (g) and root dry weight (g). Analysis of variance was done separately for the three growing conditions (T1, T2 and T3). Associations between the characters were also analysed separately for each treatment.

Results and discussion

Salinity tolerance of rice at the germination stage

It can be observed from Table 1 and Figure 1 that in all the genotypes there was a gradual decrease in germination with an increase in salinity, which supports the observations of several authors working on rice (Kaddah, 1963; Flowers and Yeo, 1981; Lutts et al., 1995). In the control, 16 genotypes showed excellent germination, ranging from 98%–100%, while all other genotypes had more than 90% germination.

Table 1
Genotypic responses of germination percentage to different levels of salinity for 27 Vibha rice genotypes

No.	Genotype	Germination (%)			
		T1	T2	T3	T4
1	VBR 611	93	96	54	5
2	VBR 612	94	93	49	1
3	VBR 614	98	89	48	8
4	**VBR 615**	**95**	**99**	**73**	**44**
5	VBR 616	99	78	22	0
6	VBR 618	100	94	62	17
7	VBR 619	95	66	57	10
8	VBR 620	96	46	14	6
9	VBR 621	93	63	46	9
10	VBR 622	99	100	68	27
11	VBR 623	98	85	46	15
12	VBR 624	99	96	65	26
13	VBR 625	91	37	18	0
14	VBR 628	100	71	30	9
15	VBR 629	98	99	75	13
16	VBR 630	98	89	56	12
17	VBR 631	88	14	12	3
18	VBR 632	98	95	51	18
19	VBR 633	100	96	64	17
20	VBR 634	95	48	19	1
21	VBR 636	100	98	63	13
22	**VBR 638**	**100**	**100**	**93**	**46**
23	VBR 640	100	93	53	8
24	VBR 642	95	65	33	16
25	**VBR 644**	**98**	**98**	**84**	**48**
26	645	98	53	64	2
27	798	91	75	7	2

T1: control; T2: 0.15 M; T3: 0.20 M; T4: 0.25 M NaCl

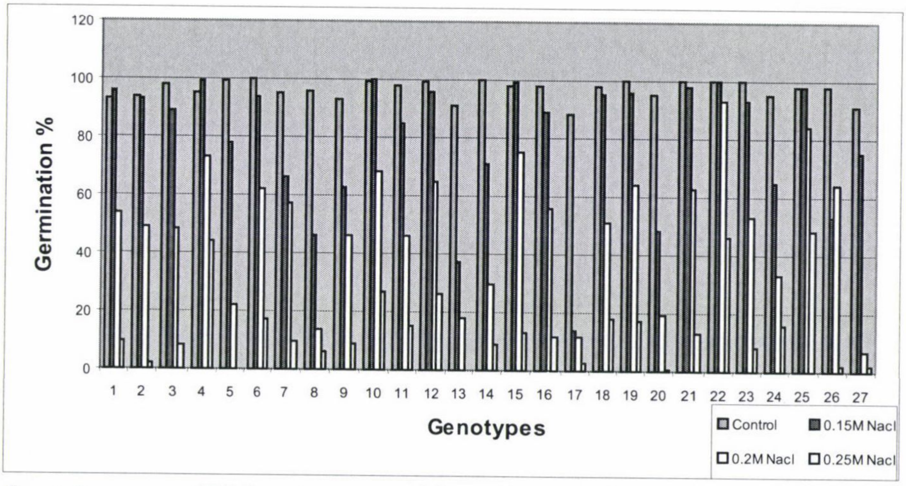

Fig. 1. Responses of 27 rice genotypes to different levels of salinity at the germination stage

In general, except for a few genotypes, very little difference was found from the control at T2, and 13 genotypes showed very good germination, ranging from 93% to 100%. At T3 only four genotypes could be considered as tolerant, namely VBR 638 (93%), VBR 644 (84%), VBR 629 (75%) and VBR 615 (73%). With the exception of VBR 629, these genotypes could also be regarded as tolerant at T4, though the germination percentage was reduced to a considerable extent for all the genotypes. At 0.25 M NaCl, 13 genotypes that showed less than 10% germination can be considered as highly susceptible, as can be seen in Table 1.

Variability, heritability and character association of rice genotypes at the seedling stage

Salinity tolerance based on character means

Assuming that all the genotypes showed normal expression under control condition (T1), deviations from the control due to saline conditions (T2 & T3) were used to determine the salinity tolerance of each genotype. All 29 genotypes were given a score for each character (shoot length, root length, shoot dry weight, root dry weight) based on the deviation value in ascending order, where 1 indicated the genotype having the smallest deviation from T1 to T2 and from T1 to T3 and 29 the genotype having the greatest deviation. The total scores of each genotype for the four characters were taken as a measure of its salinity tolerance potential. Based on the total scores, the genotypes were grouped in three categories: tolerant (up to 50); moderately tolerant (from 51–70) and susceptible (71 and above).

In the case of 0.15 M NaCl (T2), a total of 12 genotypes showed good tolerance, among which VBR 616 was the best genotype, followed by VBR 628, VBR 645, VBR 640, VBR 611, VBR 620, VBR 612, VBR 618, VBR 644, VBR 629, VBR 625 and VBR 630. Eight of the genotypes showed moderate tolerance at T2 (VBR 642, VBR 622, VBR 614, VBR 623, VBR 634, VBR 615, VBR 633 and VBR 643). The remaining nine rice genotypes were susceptible and their growth became stunted under low salinity conditions (T2). The results are presented in Table 2.

In the case of T3 (0.20 M NaCl), a total of nine genotypes showed a good level of tolerance, while twelve were moderately tolerant and eight were susceptible (Table 3). Seven of the genotypes showed tolerance in both the salinity treatments and four were moderately tolerant in both treatments. Five genotypes showed susceptibility in both treatments.

Among the 29 genotypes, VBR 616 was the best in terms of salinity tolerance (both under T2 and T3) followed by VBR 628.

Table 2
Scoring and grouping of rice genotypes under moderate (T2) saline conditions

Genotypes	Shoot length	Root length	Shoot dry weight	Root dry weight	Total score	Position	Groups
VBR 616	5	10	12	2	29	1	I Tolerant
VBR 628	2	13	7	10	32	2	
VBR 645	6	2	5	19	32	3	
VBR 640	14	3	10	16	43	4	
VBR 611	22	15	2	5	44	5	
VBR 620	16	4	4	20	44	6	
VBR 612	10	8	3	27	48	7	
VBR 618	18	16	6	8	48	8	
VBR 644	4	11	29	4	48	9	
VBR 629	3	19	16	11	49	10	
VBR 625	7	25	15	3	50	11	
VBR 630	1	20	17	12	50	12	
VBR 642	8	7	19	18	52	13	II Moderately tolerant
VBR 622	9	12	13	21	55	14	
VBR 614	23	6	21	6	56	15	
VBR 623	17	17	26	1	61	16	
VBR 634	15	14	18	14	61	17	
VBR 615	26	9	22	7	64	18	
VBR 633	27	28	1	13	69	19	
VBR 643	20	1	20	29	70	20	
VBR 631	19	5	24	23	71	21	III Susceptible
VBR 632	21	18	8	24	71	22	
VBR 636	11	29	9	25	74	23	
VBR 638	12	21	28	15	76	24	
VBR 641	24	27	11	17	79	25	
VBR 624	25	23	14	22	84	26	
VBR 621	28	26	23	9	86	27	
VBR 798	13	24	25	26	88	28	
VBR 619	29	22	27	28	106	29	

The rice genotypes showed significant differences for all five characters studied (Table 4) under all three growing conditions (T1, T2 and T3). The variability of each character was measured by the range and the genotypic coefficient of variation (GCV). In the case of 1000-grain weight, shoot length, root length and shoot dry weight the difference between GCV and phenotypic coefficient of variation (PCV) was small, indicating that the environment had very little influence on the expression of these four characters; this was true for all three growing conditions. In the case of root dry weight, however, the difference between GCV and PCV was greater, indicating that the character was more influenced by the environment. This was again true for all three growing conditions. The highest GCV was found for shoot length and shoot dry weight, followed by root dry weight and 1000-grain weight, whereas root length had a much lower value of GCV compared to the other characters. This high genetic variability present in rice can be exploited for genetic improvement by selection (Burton and De Vane, 1953).

Table 3

Scoring and grouping of rice genotypes under strong (T3) saline conditions

Genotypes	Shoot length	Root length	Shoot dry weight	Root dry weight	Total score	Position	Groups
VBR 616	7	3	7	2	19	1	I Tolerant
VBR 628	5	9	9	8	31	2	
VBR 630	2	20	2	9	33	3	
VBR 611	20	11	1	5	37	4	
VBR 642	10	6	11	13	40	5	
VBR 645	3	17	5	15	40	6	
VBR 636	8	8	4	23	43	7	
VBR 644	9	4	29	4	46	8	
VBR 618	14	1	15	18	48	9	
VBR 615	19	12	14	6	51	10	
VBR 619	23	2	20	7	52	11	
VBR 629	1	7	17	28	53	12	
VBR 634	18	14	10	11	53	13	II Moderately tolerant
VBR 640	15	10	18	12	55	14	
VBR 623	25	5	28	1	59	15	
VBR 620	16	18	8	19	61	16	
VBR 632	17	13	3	29	62	17	
VBR 612	11	22	6	26	65	18	
VBR 798	13	26	12	16	67	19	
VBR 622	12	19	16	21	68	20	
VBR 614	24	16	13	17	70	21	III Susceptible
VBR 643	22	15	27	14	78	22	
VBR 638	6	23	26	24	79	23	
VBR 624	29	25	23	3	80	24	
VBR 625	4	29	22	27	82	25	
VBR 633	27	27	25	10	89	26	
VBR 621	28	21	21	20	90	27	
VBR 631	21	24	24	22	91	28	
VBR 641	26	28	19	25	98	29	

A character with high heritability can be improved through selection. Heritability estimates (broad sense) are used to determinate the proportion of the total genetic variation. High heritability was found for test weight (98%) and for shoot length under all three growing conditions (98% in T1; 95% in T2; 96% in T3). High heritability was also found for root length and shoot dry weight, though in the case of root length a comparatively low value (73%) was found at high salinity (T3), compared with 91% at T1 and 94% at T2, and for shoot dry weight the value was lower (79%) at T2 than at T1 (88%) and T3 (89%). Among the four characters that exhibited variation only root dry weight showed low heritability under all three growing conditions (T1 55%; T2 45%; T3 57%), indicating that under saline conditions the genotypes maintain root length and root weight by a process of osmotic adjustment.

Table 4

Analysis of variance under control (T1), moderately saline (T2) and strongly saline (T3) conditions

Characters	GC	MSS	Mean	Range	GCV	PCV	H (bs)	GA
1000-GW	T1	0.5439 **	19.45	12.00–25.17	21.86	21.98	0.98	8.71
Shoot length	T1	0.4711 **	13.18	8.17–20.67	30.01	30.20	0.98	8.10
	T2	0.2632 **	10.49	7.00–17.83	28.04	28.63	0.95	5.93
	T3	0.1716 **	8.99	5.01–25.17	26.43	26.92	0.96	4.81
Root length	T1	0.5959 **	12.11	9.00–14.17	11.47	11.97	0.91	2.74
	T2	0.7417 **	10.57	8.03–13.33	14.29	14.75	0.94	3.11
	T3	0.8117 **	10.23	7.02–13.17	16.01	16.23	0.73	3.33
Shoot dry weight	T1	0.3368 **	0.11	0.06–0.17	29.07	30.88	0.88	0.06
	T2	0.3087 **	0.10	0.05–0.18	29.67	33.25	0.79	0.06
	T3	0.2280 **	0.09	0.05–0.14	29.22	31.03	0.89	0.05
Root dry weight	T1	0.3106 **	0.04	0.02–0.06	24.53	33.50	0.54	0.01
	T2	0.2744 **	0.03	0.02–0.05	23.44	34.78	0.45	0.01
	T3	0.3151 **	0.03	0.01–0.05	27.78	36.77	0.57	0.01

GW: grain weight; GC: growing conditions

Character association

The 1000-grain weight exhibited a positive and significant correlation with shoot length (T1: 0.83, T2: 0.86, T3: 0.77) and shoot dry weight (T1: 0.92, T2: 0.96, T3: 0.91) under all three growing conditions. and a very low, insignificant positive correlation with root length (T1: 0.23, T2: 0.11, T3: 0.30). This trait showed a varying degree of correlation with root dry weight under different growing conditions, having high correlation coefficients at T3 (0.86) and T1 (0.73), but only a low positive correlation at T2 (0.59). Seedling dry weight showed a positive correlation with shoot length under all three growing conditions (T1: 0.80, T2: 0.90, T3: 0.81), but a comparatively low correlation with root dry weight at T1 (0.63) and T2 (0.51), though there was a significant positive correlation at T3 (0.82) (Table 5). This reflects the fact that under strong salinity root growth is stimulated to allow the absorption of more water as a mechanism of osmotic adjustment.

Conclusions

The selection of rice tolerant to salinity at the germination and seedling stages is highly desirable for good seedling establishment. The results of these two experiments strongly support the observations of other authors that increasing salinity reduces germination and seedling growth and affects crop growth in rice at different growth stages (Pearson and Bernstein, 1959; Kaddah, 1963; Flowers and Yeo, 1981; Lutts et al. 1995). As observed in the present study, significant variability in seedling growth was reported in rice (Zafar et al., 2004).

Table 5
Genotypic correlation coefficient between different characters under three growing conditions

Characters	Growing conditions	Shoot length	Root length	Shoot dry weight	Root dry weight
1000-grain weight	T1	0.83	0.23	0.92	0.73
	T2	0.86	0.11	0.96	0.60
	T3	0.77	0.30	0.91	0.86
Shoot length	T1		0.36	0.80	0.37
	T2		0.16	0.90	0.57
	T3		0.43	0.81	0.62
Root length	T1			0.16	0.35
	T2			0.03	0.23
	T3			0.19	0.34
Shoot dry weight	T1				0.63
	T2				0.51
	T3				0.82

In the present study, only four genotypes had germination ranging from 73–93% at 0.2 M NaCl, and could thus be considered as resistant; these were VBR 638 (93%), VBR 644 (84%), VBR 629 (75%) and VBR 615 (73%). With the exception of VBR 629, these genotypes were also resistant at 0.25 M NaCl. At the seedling stage, a total of 12 genotypes exhibited tolerance to 0.15 M NaCl (T2), namely VBR 616, VBR 628, VBR 645, VBR 640, VBR 611, VBR 620, VBR 612, VBR 618, VBR 644, VBR 629, VBR 625 and VBR 630. This shows that, except for a few genotypes, salinity tolerance at the germination stage is not generally correlated with tolerance at the seedling stage. In rice very few studies have been made on the mechanism of tolerance of salinity in rice. In sorghum, osmotic adjustment and the ion and solute contents play an important role under sodium chloride stress (Khan et al., 1997). Root length and root dry weight exhibited little or no reduction at higher salinity levels, probably due to osmotic adjustment.

The results suggest that high genetic variability and the high heritability of some of the traits offer great scope for the genetic improvement of rice cultivars for salinity tolerance.

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INFLUENCE OF BIOFERTILIZERS AND ORGANIC AMENDMENTS ON NITROGENASE ACTIVITY AND PHOTOTROPHIC BIOMASS OF SOIL UNDER WHEAT

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The role of blue green algal (BGA) biofertilizers has been limited to its relevance and utilization in rice crops, and scanty information is available on their use in conjunction with organic amendments and their influence on wheat *(Triticum aestivum)*. An experiment was conducted from November 2003 to April 2004 in the fields of the Indian Agricultural Research Institute (IARI), New Delhi, India to evaluate the effect of vermicompost, farmyard manure and biofertilizers (BGA and *Azotobacter*) in different combinations with chemical fertilizers ($N_{40}P_{30}K_{30}$) in wheat (var. HD 2687). Selected soil biological parameters (cyanobacterial diversity/abundance, nitrogenase activity and the phototrophic biomass of soil cores) were measured. The application of vermicompost in combination with BGA biofertilizer ($B+V+N_{40}P_{30}K_{30}$) brought about a significant increase in nitrogenase activity (from 0.1 in $N_{80}P_{30}K_{30}$ to 2.0 nmoles mg chl^{-1} h^{-1}), while *Azotobacter* + BGA ($+N_{40}P_{30}K_{30}$) treatment gave the highest values of chlorophyll (1.19 μg g^{-1} soil). The addition of vermicompost and farmyard manure ($+N_{40}P_{30}K_{30}$) enhanced cyanobacterial abundance, and cyanobacterial genera such as *Nostoc*, *Anabaena*, *Calothrix, Oscillatoria* and *Phormidium* were the dominant forms observed under the wheat crop. The synergistic effect of organic amendments, biofertilizers and chemical fertilizers, especially BGA inoculants, advocates their utilization in wheat crops to improve soil fertility.

Key words: ARA, *Azotobacter,* biofertilizer, chlorophyll, cyanobacteria, FYM, vermicompost, wheat

Introduction

The role of organic farming has been much emphasized in agriculture, but a comprehensive evaluation of its efficiency and applicability in Asian countries, especially in India, is still lacking. Chemical fertilizers are today an indispensable part of agricultural practices, but their increasing application is neither environment-friendly, nor economically viable on a long-term basis

(Choudhury and Kennedy, 2004). Traditional compost/manures and biofertilizers are two major components of organic farming, which offer an economically attractive and ecologically sound means of reducing external inputs and improving internal resources (Saxena and Tilak, 1994; Pathak et al., 1997). However, the paucity of real-time scientific data and the existence of socio-cultural constraints have limited the use of such alternatives in modern agriculture.

Cyanobacteria or blue green algae (BGA) have long been known to be one of the most important organisms responsible for maintaining and improving the productivity of rice fields (Roger et al., 1993). The significance of nitrogen-fixing BGA in the self-maintenance of the status of tropical rice field soils and its application as inoculant has been found to bring about a yield improvement of 5–25% in rice, even in the presence of high doses of nitrogenous fertilizers (Venkataraman, 1981; Yanni, 1992). Wheat is an important crop, in which high doses of fertilizers are required to obtain high yields. However, biofertilizers such as BGA have not been exploited as supplementary inputs in this crop and very scanty information is available on the use of BGA biofertilizers along with *Azotobacter*, which is a well-established inoculant for this crop (Lakshminarayana et al., 1992).

Organic inputs such as vermicompost have been exploited commercially in horticultural crops to improve plant nutrient availability and soil microbial activity (Edwards, 1995), and the higher crop yields obtained have been attributed to better physical soil structure, the presence of plant growth hormones, higher levels of soil enzymes and greater microbial populations. However, their role in a rice–wheat cropping system has received less attention. Farmyard manure is a traditional manure/compost which, since ancient times, has been applied to soil before sowing, to improve soil health and fertility (Singh et al., 1981; Tanaka, 1978). However, these vital farm inputs have yet to enter the mainstream of plant nutrient supply systems on a large scale.

The importance of phototrophic N-fixation and the role of BGA in tropical rice fields have been investigated mainly with the emphasis on ARA (acetylene-reducing activity, as an index of nitrogenase activity) and total N (Venkataraman, 1981; Kulasooriya, 1998). The influence of BGA on soil fertility in terms of phototrophic biomass can be a useful parameter in assessing soil fertility, as these organisms contribute directly and indirectly by stimulating the soil microflora (Roger et al., 1993). This enhancement has been evaluated in a rice crop (Nayak et al., 2004). However, as BGA biofertilizers are not routinely applied in wheat, the present investigation was aimed at evaluating the combined effect of vermicompost, farmyard manure and biofertilizers (BGA and *Azotobacter*) alone and in different combinations with chemical fertilizers, on the nitrogenase activity (measured as the acetylene-reducing activity of soil cores), phototrophic biomass (chlorophyll accumulation in soil cores) and cyanobacterial diversity/abundance in wheat (*Triticum aestivum*).

Materials and methods

Collection of soil samples

Soil samples (in the form of soil cores to a depth of 0–30 mm) were collected at the mid-crop growth phase (80–90 days after sowing, DAS) from wheat fields (plot size of 20 m^2) sown with variety HD 2687. All the treatments were performed in triplicate using a randomized block design, and were established in the fields of the Indian Agricultural Research Institute (IARI), New Delhi, India from November 2003 to April 2004. A field previously used for growing rice, followed by mungbean, was utilized for this investigation after ploughing and other routine field preparation procedures for growing wheat. The geographic location, along with the soil characteristics at the time of sowing, are given in Table 1. A list of the treatments, along with the abbreviations used in the text and figures, is given in Table 2. The rates of application of the different organic fertilizers were: *Azotobacter* as seed inoculant (10^7 cells g^{-1} carrier), BGA biofertilizer at the rate of 15 kg ha^{-1}, and Vermicompost and farmyard manure each at the rate of 5 t ha^{-1}. Control treatments included no application of chemical/organic fertilizers ($N_0P_0K_0$) and the application of the recommended dose of urea, single superphosphate (SSP) and muriate of potash ($N_{80}P_{30}K_{30}$). The biofertilizers and organic amendments were applied along with chemical fertilizers ($N_{40}P_{30}K_{30}$) two days prior to the sowing of wheat. The physico-chemical composition of the organic amendments used is given in Table 3. The soil-based BGA biofertilizer (containing a mixture of *Anabaena variabilis*, *Nostoc muscorum*, *Tolypothrix tenuis*, *Aulosira fertilissima*, *Plectonema* and *Phormidium* strains with soil as carrier) was prepared under polyhouse conditions, following standardized protocols (Venkataraman, 1981; Prasanna and Kaushik, 1998). *Azotobacter chroococcum* was used as a seed inoculant as per routine procedures (Rao, 1975). Soil cores were collected using a steel-coring device (20 mm diameter). A minimum of 12 fresh soil cores were collected from each treatment (four from each replicate plot), transferred directly to glass vials (55 ml, 150 × 20 mm) and stoppered with airtight subaseals. The depth of soil cores to be taken, the time of sampling and the methodology were standardized earlier, and found to be optimal for the parameters evaluated (Prasanna et al., 2003).

Measurement of nitrogen fixation

Acetylene reduction activity (ARA) was utilized as the index of nitrogenase activity and measured after incubation under field conditions for 3 h (Prasanna et al., 2003). Vials containing soil cores were injected with 3.5 ml acetylene (10% gas phase) after removing an equal amount of air, using airtight syringes. One ml samples were injected into a gas chromatograph, fitted with an oven containing a 2 m long column of stainless steel tubing (2 mm internal diameter) packed with Poropak N (80–100 mesh). Nitrogen gas flowing at a rate of 35 ml min^{-1} was used as the carrier, while hydrogen and air were used to produce the flame in the Flame Ionisation Detector. The oven, injector and detector were maintained at 100–120°C to allow for ionization and the detection of the ethylene produced. Commercially available standard ethylene was utilized as a standard for quantification and vials with an equivalent volume of water served as controls (Prasanna et al., 2003). Triplicate samples were taken from the mid-crop growth phase (60–70 DAS) of wheat. After ARA measurement, the soil cores were air dried in the shade, placed in an oven at 105°C and dry weights were recorded. The ARA values were expressed as C_2H_4 g^{-1} dry weight of soil h^{-1}. All values presented are the means of triplicate measurements.

Chlorophyll estimation

Triplicate samples of fresh soil cores (surface and below surface) were flooded with acetone–DMSO (dimethyl sulphoxide) mixture (1:1) at 4 ml g^{-1} soil and incubated in the dark for 3–4 days with intermittent shaking, as standardized earlier (Nayak et al., 2004). The concentration of chlorophyll *a* was determined from absorbance readings (750, 663, 645 and 630 nm) using the supernatant after centrifugation. Spectrophotometric quantification of the clear supernatants was done utilizing a Beckmann model DS spectrophotometer, employing the methodology and equations described by Scor/UNESCO (1966), with a solvent mixture (acetone–DMSO) serving as the control.

Table 1

Geographical details of experimental site and physicochemical properties of soil at the start of the experiment (December 2003)

Altitude	228.16 m above mean sea level
Latitude	28°4' N
Longitude	77°12' E
pH	7.3
EC	0.64 dS m^{-1}
Organic carbon	0.43%
Available N	156 kg ha^{-1}
Available P	22.5 kg ha^{-1}
Soil classification	
Texture	Sandy clay loam
Order	Inceptisol
Family	Udic Ustocrept

Table 2

Details of treatments and abbreviations used in the text

No.	Treatment	Abbreviations
1	$N_0P_0K_0$	–
2	$N_{80}P_{30}K_{30}$	–
3	$N_{40}P_{30}K_{30}$+*Azotobacter*	$N_{40}P_{30}K_{30}$+A
4	$N_{40}P_{30}K_{30}$+BGA	$N_{40}P_{30}K_{30}$+B
5	$N_{40}P_{30}K_{30}$+Vermicompost	$N_{40}P_{30}K_{30}$+V
6	$N_{40}P_{30}K_{30}$+farmyard manure	$N_{40}P_{30}K_{30}$+F
7	$N_{40}P_{30}K_{30}$+*Azotobacter*+BGA	$N_{40}P_{30}K_{30}$+A+B
8	$N_{40}P_{30}K_{30}$+*Azotobacter*+Vermicompost	$N_{40}P_{30}K_{30}$+A+V
9	$N_{40}P_{30}K_{30}$+*Azotobacter*+farmyard manure	$N_{40}P_{30}K_{30}$+A+F
10	$N_{40}P_{30}K_{30}$+BGA+Vermicompost	$N_{40}P_{30}K_{30}$+B+V
11	$N_{40}P_{30}K_{30}$+BGA+farmyard manure	$N_{40}P_{30}K_{30}$+B+F
12	$N_{40}P_{30}K_{30}$+Vermicompost+farmyard manure	$N_{40}P_{30}K_{30}$+V+F
13	$N_{40}P_{30}K_{30}$+*Azotobacter*+BGA+farmyard manure	$N_{40}P_{30}K_{30}$+A+B+F
14	$N_{40}P_{30}K_{30}$+BGA+Vermicompost+farmyard manure	$N_{40}P_{30}K_{30}$+B+V+F
15	$N_{40}P_{30}K_{30}$+*Azotobacter*+Vermicompost+farmyard manure	$N_{40}P_{30}K_{30}$+A+V+F
16	$N_{40}P_{30}K_{30}$+*Azotobacter*+BGA+Vermicompost+farmyard manure	$N_{40}P_{30}K_{30}$+A+B+V+F

Table 3

Nutrient composition of Vermicompost and farmyard manure used in the experiment

Nutrient	Vermicompost	Farmyard manure
N%	1.68	0.72
P_2O_5%	5.12	0.19
K_2O%	0.82	0.53
Ca%	0.43	0.90
Mg%	0.14	0.21
Fe (ppm)	180.50	148.15
Mn (ppm)	96.50	71.00
Zn (ppm)	23.95	14.35
Cu (ppm)	4.83	2.56
C:N ratio	15.30	30.86

Statistical analyses

The triplicate sets of data for the various physiological parameters were subjected to ANOVA (analysis of variance) in accordance with the experimental design (completely randomized block design) using the MSTAT-C statistical package to quantify and evaluate the source of variation. Comparison with the control treatments ($N_0P_0K_0$ serving as the residual values after rice–mungbean cropping sequence, and $N_{80}P_{30}K_{30}$, representing the recommended fertilizer control) was done for all the parameters analysed.

Identification and enumeration of culturable cyanobacteria

Standard plating techniques were used for the isolation and purification of cyanobacterial strains (Stanier et al., 1971). Identification of the strains was done using the keys published by Desikachary (1959). The most probable number technique was utilized for enumerating the nitrogen-fixing and non-nitrogen-fixing cyanobacterial populations in the selected treatments.

Results

Soil chlorophyll and ARA

On evaluating the effect of nitrogenous fertilizer (urea) and biofertilizers/organic inputs (*Azotobacter*, BGA and farmyard manure), it was observed that all the treatments were significantly superior to the control treatments ($N_0P_0K_0$, $N_{80}P_{30}K_{30}$), except the application of vermicompost or FYM alone, in terms of soil chlorophyll. FYM had a stimulatory influence on chlorophyll accumulation (Table 4) when applied in combination with BGA along with 40 kg N (as urea), and the highest value of 2.0 $\mu g\ g^{-1}$ soil was recorded in this treatment. The lowest values were recorded in the control plots where only chemical fertilizers were applied ($N_{80}P_{30}K_{30}$).

Table 4

Chlorophyll content and acetylene reduction activity of soil cores collected from wheat fields inoculated with different chemical fertilizers and biofertilizers

Treatments	Chlorophyll ($\mu g\ g^{-1}$ soil)	ARA (nmol $C_2H_4\ g^{-1}$ soil)
$N_0P_0K_0$	0.03	0.420
$N_{80}P_{30}K_{30}$	0.02	0.493
$N_{40}P_{30}K_{30}$+A	0.667	0.509
$N_{40}P_{30}K_{30}$+B	0.543	0.674
$N_{40}P_{30}K_{30}$+V	0.020	0.842
$N_{40}P_{30}K_{30}$+F	0.010	0.512
$N_{40}P_{30}K_{30}$+A+B	0.470	1.027
$N_{40}P_{30}K_{30}$+A+V	1.187	0.626
$N_{40}P_{30}K_{30}$+A+F	0.430	0.512
$N_{40}P_{30}K_{30}$+B+V	0.217	0.568
$N_{40}P_{30}K_{30}$+B+F	2.00	0.451
$N_{40}P_{30}K_{30}$+V+F	0.113	0.561
$N_{40}P_{30}K_{30}$+B+V+F	0.260	0.861
$N_{40}P_{30}K_{30}$+A+V+F	0.560	0.637
$N_{40}P_{30}K_{30}$+A+B+F	0.250	0.844
$N_{40}P_{30}K_{30}$+A+B+V+F	0.787	0.742
CD(P=0.001)	0.124	0.144

For treatment codes, see Table 2

The measurement of ARA (nmoles C_2H_4 g^{-1} soil h^{-1}), as an index of nitrogenase activity, indicated that soil cores taken from the $N_{40}P_{30}K_{30}$+A+B plots had the highest value, followed by $N_{40}P_{30}K_{30}$+B+V+F, with the lowest value for $N_0P_0K_0$ (Table 4**)**. The $N_{40}P_{30}K_{30}$+B+V+F and $N_{40}P_{30}K_{30}$+A+B+F treatments also enhanced ARA values. The combination of all four organic inputs (A+B+F+V) was also stimulatory. The B+F combination gave low ARA values.

Cyanobacterial abundance

Plots treated with chemical fertilizers alone ($N_0P_0K_0$, $N_{80}P_{30}K_{30}$) or in combination with biofertilizer/organic inputs (*Azotobacter*, BGA, Vermicompost and FYM) showed a wide range of values for cyanobacterial abundance and diversity. The highest cyanobacterial abundance was recorded in plots treated with urea (N_{40}) in combination with BGA+A+F+V, followed by $N_{40}P_{30}K_3$+A+B+F (Fig. 1). The population count varied from 0.9–1.6 $\times 10^5$ g^{-1} soil (for N_2-fixers) to 1.2–2.6 $\times$ 10^5 for total BGA (including non-nitrogen-fixing and nitrogen-fixing BGA). The lowest values were recorded for samples from control plots.

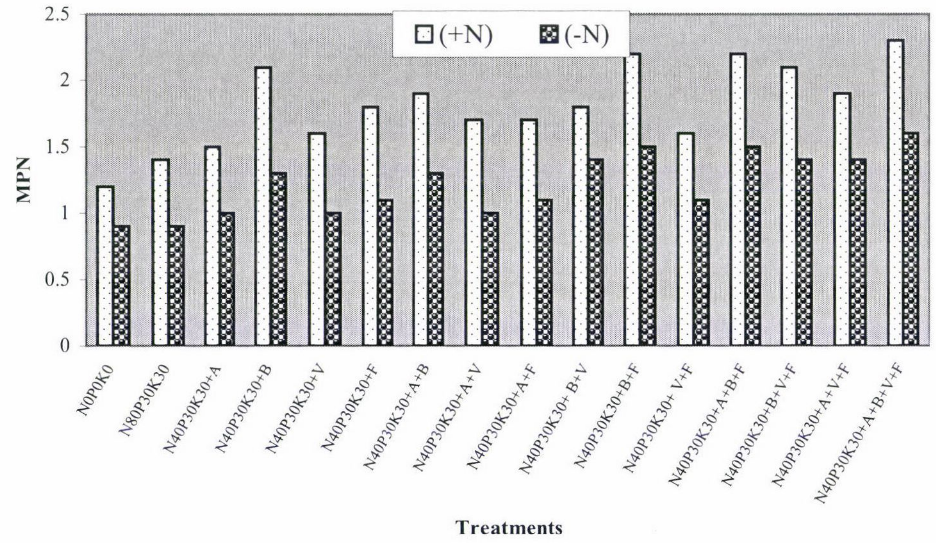

Fig. 1. Enumeration of cyanobacterial population ($\times 10^5$) in the various treatments by the MPN (Most Probable Number) technique, using nitrogen-supplemented (+N) and nitrogen-deficient (-N) media (For treatment codes, see Table 2)

Studies carried out on the relative distribution of BGA in stationary enrichment cultures on liquid/agar-based medium, both in nitrogen-supplemented and nitrogen-deficient medium, using soil samples from the different treatments, indicated that the number of genera proliferated throughout the crop season. In nitrogen-deficient medium, *Nostoc* was the first to appear and remained the most abundant genus, followed by *Anabaena* and *Calothrix* towards the later stages. Interestingly, *Nostoc* remained the dominant form in medium supplemented with nitrogen, as did *Phormidium*, followed by *Anabaena* and *Oscillatoria* in decreasing order of abundance. A total of seven heterocystous forms (belonging to genera *Nostoc, Anabaena* and *Calothrix*) and two non-heterocystous forms (belonging to genera *Phormidium* and *Oscillatoria*) were isolated and purified.

Discussion

The key factor in the success of integrated nutrient management is an efficient and economic supply of N, the element required in the largest quantity in comparison with other essential ones. It is well known that the utilization efficiency of N from fertilizer sources is very low, as it is lost from the soil through various chemical and biological processes. Also, the increasing use of nitrogenous fertilizers is neither economically nor environmentally viable, hence biological alternatives need to be explored to maintain soil fertility. Biofertilizers are environment-friendly, low-cost agricultural inputs, which play an important role in enhancing crop productivity through nitrogen fixation, phosphate solubilization and the production of plant growth-promoting compounds and biocidal compounds capable of inhibiting pathogens/pests in the soil. Organic amendments, on the other hand, especially farmyard manure, have been traditionally employed as a routine practice, applied basally before the sowing/transplanting of crops and bringing about a definite improvement in soil properties (Houng, 1976; Tanaka, 1978). They have low agronomic value, on a short-term basis, but play a crucial role in the sustainability of soil fertility, despite problems related to N immobilization. Vermicompost, or earthworm casts used as compost, have been shown to enhance crop performance due to several agronomic benefits, including their significant role as slow-release natural fertilizers (Edwards, 1995; Pashanasi et al., 1996). Vermicompost is an efficient source of plant nutrients and the slower rate of N release from these manures gives it an advantage over compost or synthetic fertilizers

In the present investigation, the positive synergistic effect of biofertilizers and organic amendments (Vermicompost and FYM) was observed on two significant soil fertility parameters: N accumulation in the soil as a result of nitrogenase activity, i.e. biological nitrogen fixation, measured as acetylene-reducing activity, and photosynthetic biomass (using chlorophyll accumulation as an index). The application of *Azotobacter* and BGA ($+N_{40}P_{30}K_{30}$) enhanced soil chlorophyll significantly, although the highest values were recorded for *Azotobacter* + vermicompost (+$N_{40}P_{30}K_{30}$), followed by treatments involving farmyard manure in combination with BGA + *Azotobacter* + vermicompost. The application of vermicompost and conventional compost was shown earlier to improve soil microbial activity and plant nutrient availability (Chaoui et al., 2003). It can be surmised that FYM stimulates the growth of photosynthetic microorganisms in the soil, leading to increased biomass measured as soil chlorophyll.

The positive effects of the application of biofertilizers (BGA and *Azolla* biofertilizers) on soil chlorophyll were also observed in earlier studies in rice (Nayak et al., 2004). In the present investigation, the application of FYM enhanced cyanobacterial abundance in the field, which may indirectly improve soil fertility by stimulating the surrounding microflora in the soil. Although this is a less investigated aspect, it could be a reliable indicator of photosynthetic biomass and could therefore indirectly indicate the abundance of photoautotrophic microbial populations, which are the primary producers in the soil.

Acetylene-reducing activity, used as an index of biological nitrogen fixation, was highest in treatments involving BGA and *Azotobacter*, while BGA + Vermicompost + FYM application gave the next highest values. The lowest values were recorded in the controls ($N_{80}P_{30}K_{30}$; $N_0P_0K_0$) and for FYM. This could be indicative of the low abundance of diazotrophs in the native soil. The application of bioinoculants (BGA and *Azotobacter*) not only leads to their establishment in soil, but also stimulates the native flora, leading to high nitrogenase activity, as measured in the soil cores. Also, the combined application of biofertilizers and organic composts gave significantly higher values of ARA, as compared to their application alone. The application of soil amendments such as Vermicompost and FYM is known to stimulate microbial populations in several crops, of which rice has been thoroughly investigated (Aiyer, 1963), and it was observed that the establishment of *Azotobacter* was enhanced following inoculation with blue green algae.

The addition of nitrogenous fertilizers is known to have a significant influence on ARA (Singh et al., 1981; Yanni, 1992) and the deep placement of fertilizers in the soil is recommended to obtain benefits from both chemical inputs and biological nitrogen fixation (BNF). It is well documented that the application of nitrogenous fertilizers, especially urea, at the rate of 30–60 kg N ha^{-1} does not adversely affect ARA (Venkataraman, 1981; Nayak et al., 2004).

It is well established that biological nitrogen fixation (BNF) plays a positive role in the sustainability of agriculture in the present era, by improving the N economy of soils. Much of the research on BNF has involved identifying efficient strains for use as inoculants, especially in relation to the legume–*Rhizobium* symbiosis. *Azotobacter* was first used for legumes in the USSR and used to treat the seeds of wheat, barley, maize, sugar beet, etc.

(Lakshminarayana, 1993). Later work showed that 10–20 kg N ha^{-1} can be saved through the use of *Azotobacter,* which also produces plant growth regulatory substances (Rao, 1975). Three doses of FYM were tested along with *Azotobacter* inoculants and 60 kg N, leading to a net saving of 60 kg N ha^{-1} (Zambre et al,. 1984). Although, among field crops, wheat was shown to be problematic for the proliferation of native and inoculated *Azotobacter*, later investigations showed that *Azotobacter* performed better at lower levels of nitrogen (0–30 kg N ha^{-1}) and contributed a 35–50% net saving in N fertilizer (Zambre et al., 1984). Although in the present study population counts of *Azotobacter* were not taken, the increased ARA values may be reflective of their contribution towards N-fixation. In this investigation, *Azotobacter* inoculation, applied alone or in combination with farmyard manure, vermicompost or BGA, enhanced ARA. This is a significant indication of its compatibility with organic composts and BGA.

Reports on the use of BGA biofertilizers in conjunction with wheat are meagre. From an agronomic point of view, BGAs are thoroughly investigated phototrophic systems which, besides their N-enrichment potential, increase the water-holding capacity, porosity, structure and cation exchange capacity of the soil (Venkataraman, 1970; Kulasooriya, 1998). The enhancement of ARA has been observed in soil cores from rice crops to which BGA supplemented with 30/60 kg N ha^{-1} was applied (Prasanna et al., 2003; Nayak et al., 2004), and estimates of N_2-fixation at 45 DAT (days after transplanting) ranged from 7.5–177.0 kg N ha^{-1}, through extrapolation using standard procedures (Alimagno and Yoshida, 1977). Using similar procedures in the present investigation, the rates of N fixed ranged from 0.5–110 kg N ha^{-1}.

As all the treatments involving a combination of a minimal dose of chemical fertilizers along with soil amendments and biofertilizers were significantly superior in terms of the parameters investigated, these can be advocated as suitable treatments in wheat crops for the significant enhancement of N accretion in the soil through biological nitrogen fixation, leading to greater crop yields.

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COMPARISON OF THE GRAIN YIELD AND QUALITY POTENTIAL OF MAIZE HYBRIDS IN DIFFERENT FAO MATURITY GROUPS

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A total of 96 hybrids from four maturity groups (FAO 200, 300, 400, 500) were tested in two years (2006, 2007) at two locations in Hungary (Martonvásár, Szarvas). Considerable differences were found between the years for the grain yield per hectare and for the grain quality parameters. In 2006 record yields were achieved at both locations, averaging 11.61 t/ha in Martonvásár and 12.20 t/ha in Szarvas, due primarily to well-timed irrigation in Martonvásár and to good rainfall supplies in Szarvas. In 2007 both locations suffered from drought, with less rainfall than average during the critical months of the vegetation period, which was partially compensated for by irrigation in Martonvásár, giving a yield average of 5.96 t/ha, while the hybrids grown in Szarvas had a yield average of 5.06 t/ha. The grain quality parameters exhibited a close correlation with the grain yield in the individual FAO maturity groups. Hybrids of the flint type, which have a short vegetation period, had high protein and oil contents, but the yield averages were low due to the slower rate of starch incorporation. Hybrids of the dent type have a longer vegetation period and more intense carbohydrate accumulation, but low protein and oil contents. In wet years and locations there was a higher rate of starch accumulation, while dry years are favourable for protein and oil accumulation. The Bravais correlation coefficient was calculated between the yield and the grain quality parameters (averaged over years, locations and varieties). A positive, moderately strong correlation (0.68) was found between the yield and the starch content, a negative, moderately strong correlation (–0.52) between the yield and the protein content, and a loose negative correlation (–0.19) between the yield and the oil content.

Key words: maize hybrid, water, FAO maturity group, yield, protein, oil and starch content

Introduction

Although maize is of tropical origin, the highest yields are not recorded in the tropics, but in the temperate zone, where both the rainfall and the temperature are adequate (Menyhért, 1979). Under Hungarian conditions the quantity and distribution of rainfall during the vegetation period are unfavourable. When analysing the agrometeorological conditions required for maize production, Polerecky (1976) stated that rainfall quantities of 155–180 mm are required during the driest months of July and August if high yields are to be obtained. Berényi (1945) found that rainfall fluctuations in July had a greater influence on the grain yield than temperature variability. Gyenesné et al. (2002) recorded a close positive correlation between ear yield and the rainfall quantity in July (Hegyi et al., 2005). Long-term water deficiency during flowering increased the frequency of aborted kernels in the ears (Zinselmeier et al., 1995). Quaranta et al. (2001) reported that water stress affected flowering, yield quantity and grain quality parameters. In wet locations more starch was incorporated in the grain, leading to higher yields (Hegyi et al., 2007). According to Pásztor et al. (1998) the year had no great effect on starch content, but the starch and fat contents increased with the thousand-kernel mass (TKM). Bálint (1977) found that in hybrids with lower TKM the endosperm and the starch accumulation were smaller, while the grain protein content was relatively greater. In field production, greater per hectare maize yields generally have a lower protein content, while varieties containing 12% or more protein usually have lower yields (Sprague, 1977). Similar findings were reported by Svecnjak et al. (2007), who revealed negative correlations between the grain yield and the grain protein content ($r = -0.48^*$) and oil content ($r = -0.19$). Improvements in protein content and quality would be of benefit both for animal feeding and human consumption (Pásztor and Győri, 1991; Mertz, 1992). Among the chemical factors influencing grain quality, the protein and oil content can be considerably influenced by breeding (Bálint, 1977), the starch content primarily by selection. No heterosis has been observed for protein and oil content; hybrid progeny had values less than the parental mean for these parameters (Gyenesné-Hegyi et al., 2001; Pepó et al., 2007). In wet years maize hybrids had lower protein contents than in dry years (Prokszáné Paplogó et al., 1995; Gyenesné-Hegyi et al., 2001, 2002). Irrigation had a significant effect on the protein and oil concentrations (Josipovic et al. (2007).

Materials and methods

An experiment was set up at two locations in Hungary (Martonvásár, Szarvas) in 2006 and 2007 in a randomised block design with four replications. At each location 24 hybrids were tested from each of four maturity groups (FAO 200, 300, 400, 500). The experiments were sown and harvested mechanically. Prior to harvest, five sample ears were taken from each plot, and the yield average per hectare of each hybrid was corrected based on the ear mass of the sample ears. Evaluations were made of the yield average (t/ha), followed by chemical analysis to determine the protein, oil and starch contents of the kernels. The measurements were made using a Fourier transform NIR spectrometer (Bruker, Ettlingen, Germany).

Rainfall conditions differed between both the locations and the years (Table 1). In Martonvásár there was less rainfall than average during the vegetation period in both years, with a critical rainfall deficiency during flowering in July (only 10 mm of rain and 23 very hot days in July in 2006; 25 mm and 13 very hot days in 2007). Irrigation water equivalent to 80 mm rainfall was applied during flowering in both years to reduce the atmospheric drought. In Szarvas rainfall exceeded the many years' average during the vegetation period in both years, but in July 2007 the rainfall during flowering was 17 mm less than average, with 15 very hot days. The rainfall deficiency, combined with atmospheric drought, contributed to poor fertilisation.

Table 1

Rainfall conditions at the two locations during the vegetation period (Apr.–Sep.) in the experimental years

Locations	Rainfall during the vegetation period, mm					
	2006	30-year mean	Δ	2007	30-year mean	Δ
Martonvásár	246.5	312.0	–65.5	265.0	312	–47.0
Szarvas	393.7	313.0	80.7	367.2	313.0	54.2

Results and discussion

In 2006 the grand mean for the yield of maize hybrids in Martonvásár was 11.61 t/ha, compared with 12.20 t/ha in Szarvas. Although there was less rainfall than average during the vegetation period in Martonvásár, the distribution was favourable, and the plots were irrigated during flowering. In Szarvas there was above-average rainfall, so record yields were achieved without irrigation. There was a significant difference between the locations. At both locations the greatest yields were recorded for varieties in the FAO 400 maturity group (12.58 t/ha in Szarvas; 12.52 t/ha in Martonvásár; Fig. 1). In the droughty year of 2007 there was less rainfall than average in Martonvásár throughout the vegetation period, with the exception of August and September. The irrigation water applied in July moderated the drought damage. In Szarvas there was more rain than usual during the vegetation period in 2007, but the distribution was unfavourable. The rainfall deficiency in July, coupled with very hot days, led to poor fertilisation and lower yields. The grand mean for the maize yield was 5.96 t/ha in Martonvásár and 5.06 t/ha in Szarvas. In Martonvásár the highest yields were obtained in maturity groups FAO 400 (5.93 t/ha) and FAO 500 (7.74 t/ha). Due to the longer vegetation period these hybrids were able to develop two ears in response to the 112.2 mm of rainfall during the first ten days of August. In Szarvas the highest yields were recorded for the FAO 300 hybrids (5.40 t/ha), while the hybrids in FAO maturity groups 200 and 500 both had average yields of 4.81 t/ha.

The grain quality parameters (starch, protein and oil content) were evaluated for each FAO maturity group in both years (Table 2). In 2006 higher yields were recorded at both locations than in 2007. Consequently the starch content was higher in 2006 (averaging 72.42% in Martonvásár and 72.98% in Szarvas). Starch incorporation was lowest at both locations in the FAO 200 maturity group, dominated by flint and semi-dent hybrids, which have smaller TKM than the later maturing dent types. The greatest starch accumulation was recorded for the FAO 500 hybrids (Martonvásár: 73.74%, Szarvas: 73.87%). In 2007 the starch accumulation in the grains was significantly lower due to the drought (Martonvásár: 70.71%, Szarvas: 72.04%). In Martonvásár the starch content was lowest in the FAO 200 group (68.40%), which averaged 71.18% in Szarvas. Due to the smaller yield averages, both the FAO 200 and FAO 300 hybrids had low starch contents. The greatest quantities of starch were measured in the dent hybrids in the FAO 500 group (72.20% and 73.47% in Martonvásár and Szarvas, respectively).

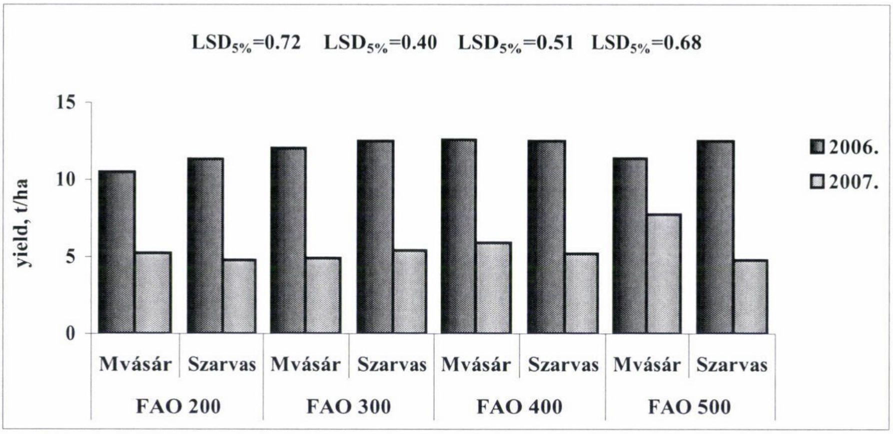

Fig. 1. Yields of maize hybrids in different FAO maturity groups, locations and years, averaged over the varieties (t/ha)

In 2006, the highest starch contents were observed for the hybrids Mv Tarján (73%), Norma (73%) and Mv 355 DMSC (73%) in the FAO 300 maturity group, for Koppány (74%) in the FAO 400 group and for Maxima at both locations (75%) in the FAO 500 group. In 2007, the highest starch contents were once again observed for Mv Tarján (72%) in the FAO 300 group and Mv Koppány (73%) in the FAO 400 group, while in the FAO 500 group the greatest values were found for Kámasil (74%) and Silóking (74%).

The protein and oil contents exhibited an opposing tendency to the starch accumulation, the highest values being recorded in early maturing hybrids. Starch is accumulated in the endosperm, which also contains 80% of the total protein content, so one can only accumulate at the expense of the other. In 2006 the highest protein and oil contents were recorded in the drier Martonvásár location in the FAO 200 hybrids (8.46%, compared with 8.07% in Szarvas). The grain oil content was closely correlated with the protein content, with maximum values of 3.78% and 3.87% in the FAO 200 hybrids at the two locations. As the vegetation period became longer, there was a decline in the protein and oil contents, with protein contents of 7.30% and 6.90% in the FAO 500 hybrids, and oil contents of 3.52% and 3.58% in Martonvásár and Szarvas, respectively. The oil is found chiefly in the embryo, and late-maturing hybrids with a large accumulation of starch in the endosperm generally have a smaller embryo. In the drier year of 2007 the protein and oil ratios were higher, with average protein contents of 9.89% and 9.03% and oil contents of 3.96% and 4.90% in the FAO 200 hybrids in Martonvásár and Szarvas, respectively. With an increase in the vegetation period there was a drop in protein and oil contents, with values of 7.58% and 7.35% for protein and 3.68% and 4.25% for oil in the FAO 500 hybrids in Martonvásár and Szarvas, respectively.

In 2006, which was the wetter year, the greatest protein contents were recorded for Mv 251 (10.20%) and Bodrog (10.50%) in the FAO 200 group, while in the FAO 300 group the experimental hybrids had outstanding protein contents. Experimental hybrids also had the highest oil contents, which exceeded 4% for Mv 3356 and Mv 356. In the dry year, 2007, the early hybrids had a higher relative protein content compared with the previous year. In the FAO 200 group the highest values were again recorded for Mv 251 and Bodrog (10.84%), while among the FAO 300 hybrids the protein contents of Hunor (12.26%), Mv Táltos (12.25%) and Mv Tarján (12.06%) were the greatest. The oil content of the varieties was also higher than in the previous year, being 4.99% for Ipoly, while the high protein content of Mv 251 was matched by high oil content (4.87%).

Table 2

Values of grain quality parameters for maize hybrids in different years, FAO maturity groups and locations, averaged over the varieties (%)

Location	Starch content, %		Protein content, %		Oil content, %	
	2006	2007	2006	2007	2006	2007
FAO 200						
Martonvásár	70.60	68.40	8.46	9.89	3.78	3.96
Szarvas	71.83	71.18	8.07	9.03	3.87	4.90
LSD$_{5\%}$	0.30		0.22		0.11	
FAO 300						
Martonvásár	72.24	70.53	8.32	9.52	3.59	3.78
Szarvas	72.30	70.61	7.89	9.78	3.77	4.82
LSD$_{5\%}$	0.25		0.21		0.19	
FAO 400						
Martonvásár	73.10	71.72	7.88	8.50	3.40	3.65
Szarvas	73.92	72.88	8.02	8.74	3.62	4.52
LSD$_{5\%}$	0.33		0.31		0.20	
FAO 500						
Martonvásár	73.74	72.20	7.30	7.58	3.52	3.68
Szarvas	73.87	73.47	6.90	7.35	3.58	4.25
LSD$_{5\%}$	0.28		0.26		0.12	
Mean Martonvásár	72.42	70.71	7.99	8.87	3.57	3.77
Mean Szarvas	72.98	72.04	7.72	8.73	3.71	4.62

Conclusions

The grain quality parameters of 96 maize hybrids were analysed in two years, in close correlation with the grain yield. As previously reported by Sprague (1977) and Svecnjak et al. (2007), starch accumulation in the kernels was found to increase with a rise in the yield average, while the protein and oil content declined. The two latter parameters are more strongly influenced by genetic factors than the starch content. It was concluded from the results that the flint and semi-dent types in the early maturing FAO 200 group had higher protein and oil contents, but lower starch contents, while the dent hybrids in the FAO 500 group, which have larger endosperms, have greater starch contents and a lower ratio of protein and oil. In agreement with Prokszáné Paplogó et al. (1995), Gyenesné et al. (2001, 2002) and Josipovic (2007) it was observed that in wet years, or as the result of irrigation, more starch was accumulated in the kernels than in dry years. In contrast to the opinion of Pásztor (1998) it was thus found that the year played an important role in determining the grain quality parameters of maize.

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EFFECTS OF ORGANIC MATTER AND TIME OF INCORPORATION ON ROOT DEVELOPMENT OF TROPICAL MAIZE SEEDLINGS

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Smallholders in the tropics add different organic materials to their crops at different times, based on the availability of materials and labour. However, the time of application could have an effect on the establishment and early growth of crops, especially their root systems, which has not yet been clearly identified. This paper presents the results of a study conducted under greenhouse conditions using soils from a field treated with three organic materials at 4 or 2 weeks before or at the planting of maize seeds, corresponding to the times that tropical smallholders apply these materials. The organic materials used were leaves of *Gliricidia sepium* and *Tithonia diversifolia* or rice straw, incorporated at a rate equivalent to 6 Mt ha^{-1}. A control treatment where no organic matter was added was used for comparison. The impact of the treatments on soil properties at the planting of maize seed and detailed root analysis based on root lengths were carried out until the last growth stage (V4). The addition of organic matter improved the soil characteristics, and the impact of adding *Gliricidia* leaves was most pronounced when incorporated 2 weeks before planting. The benefits of leaves of *Tithonia* or rice straw on soil quality parameters were clearly evident when added 4 weeks before planting. Organic matter enhanced the root number, root length, root growth rate and branching indices. All the organic materials suppressed the growth of maize roots when applied at planting, suggesting the existence of allelopathic effects, which could result in poor growth. The most benefits in terms of root growth were observed with *Tithonia*.

Key words: maize, organic matter, time of application, root growth

Introduction

The decline in soil fertility in the tropics is principally due to nutrient removal by crops and their residues for animal fodder, with minimal return to the farming systems. The non-replenishment of the removed nutrients or the non-application of optimal rates of fertilizers or other sources of organic matter result in the loss of soil fertility, with a decline in physical, chemical and

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biological soil properties (Zingore et al., 2003; Hartemink, 2006). Possible methods to overcome this problem of declining fertility include the application of chemical fertilizers, organic matter or preferably a combination of both. The inclusion of organic matter has long-term benefits (Rawls et al., 2003; De Costa and Sangakkara, 2006), and as most developing nations in the tropics have problems acquiring chemical fertilizers, due to the high prices which are beyond the reach of smallholders, the use of organic matter is often recommended for maintaining fertility and for sustainable cropping.

Smallholder systems in the tropics use a range of plant-based organic matter in arable crop production, ranging from green manures and cover crops to crop residues. The ability of these materials to provide nutrients to crops and enhance soil quality varies widely (Cobo et al., 2002; Kumar and Goh, 2002). In Asia, the most common organic matter used in arable cropping is crop residues, especially of rice (Singh et al., 2005) and other cereals, which have high C:N ratios, although green manures have a greater beneficial impact due to the lower C:N ratios (Sanchez, 1999; Whitbread et al., 2004). However, the use of any type of organic matter in the tropics results in increased growth and yields of all tropical crops (Singh et al., 2005; Sakonnakhon et al., 2005; Hartemink, 2006).

Tropical smallholders generally apply organic matter to their cropping systems prior to or at the time of crop establishment (Singh et al., 2005). However, the time of application of organic matter could have variable results on the establishment of the crop, as some organic materials, including green manures, could cause allelopathic effects on the emerging seedlings (Khanh et al., 2005). This could inhibit early root growth, which is vital for successful crop establishment, especially in tropical cereals (Welbaum et al., 2001), which are often subjected to moisture stress (Turner, 2000).

Maize (*Zea mays* L.) is the most important highland cereal in tropical humid Asia and is generally grown under smallholder subsistence farming conditions. Thus, the development of a good root system soon after establishment becomes imperative for successful crop yields, especially under marginal conditions, as water is the most limiting factor for this crop in this region (Moser et al., 2006) and could affect successful crop establishment and subsequent productivity. Studies by Sangakkara et al. (2004) identified the beneficial impact of soils planted with different green manures (*Crotalaria juncea* and *Tithonia diversifolia*) as long-term fallows on the root growth of maize seedlings. However, research does not indicate the impact of the time of organic matter incorporation, either in the form of green manures or crop residues, on the early root growth of crops such as maize grown in these soils, due to the time and labour involved. Thus, studies were carried out in a greenhouse to determine the impact of incorporating green manures or crop residues to the soil at different times on the root development of maize seedlings. Emphasis was placed on the initiation, development and branching patterns of the roots of maize seedlings, which have a significant impact on the successful establishment of the crop under field conditions.

Materials and methods

The experiment was carried out over the period May–August, 2004 in the greenhouse of the Department of Crop Science, University of Peradeniya, Sri Lanka. The environmental conditions over the period of study were as follows: mean daily temperature 28.9°C ± 1.98°C, humidity 78.4% ± 3.5% with an 11–12-hour day length. The shade provided by a green nylon net intercepted 25% of the incident radiation.

The soil used for the study was an Ultisol Rhododhult (Panabokke, 1996) obtained from the University Farm, located 20 km away from the main campus. The soil was prepared under field conditions at the University Farm by clean weeding and incorporating fresh leaves of either *Gliricidia sepium,* a popular tree legume, the leaves of which are used as a green manure, *Tithonia diversifolia*, a non-legume green manure, or rice straw at the rate of 6 Mt ha^{-1} (600 g m^{-2}) to an average depth of 50 cm two weeks, one week or just prior to transporting the soil to the site of experimentation. One plot was left bare to obtain soils for the control treatment. At the time of transportation, the soil of each treatment was dug to a depth of 50 cm, and mixed well after all stones were removed. The experiment thus had the following treatments: three types of organic matter added on three occasions with a control treatment with no organic matter.

In the greenhouse, the soils were filled into PVC tubes (10 cm diameter, 50 cm tall) within 24 hours of transportation at a rate of 5 kg per tube. On the same day, two pre-germinated seeds of maize (cultivar Bhadra) were planted in each tube, and watered at a rate of 200 ml per tube once every two days to exclude moisture stress. No additional fertilizer was added. The seedlings were thinned to one plant per tube at 3 days after planting. The data collected from each soil type were as follows:

At planting the bulk density, pH (1:2.5 H_2O), cation exchange capacity (CEC), organic carbon (Walkley-Black method), nitrogen (MicroKjeldhal) and available P (Olsen) were measured for five randomly chosen replicates of each soil type (SSSA, 1986)

Five days after planting, soils of three tubes per replicate were carefully washed and the seedlings removed. The number of roots and the total root length were determined using the grid technique (Tennant, 1975)

At 10, 15, 20 and 25 days after planting, which corresponded to the V1, V2, V3 and V4 growth stages, three tubes per replicate were selected randomly, the soils were carefully removed and the roots of the seedlings were separated into primary, secondary and nodal roots. The total root length (Tennant, 1975) of each category was measured. At the final sampling, the dry weight of each category of roots was obtained after drying for 24 h at 80°C. These data were used to calculate the rate of root elongation (regression analysis using logarithmic scales) and the specific root length.

The lengths of all root categories obtained at the last sampling (V4 stage) were used to derive the branching index (length of secondary and nodal roots/length of primary root), which indicates the branching pattern of the secondary roots in relation to the primary root, as described by Tsuji et al. (2005).

The factorial experiment thus had four soil types (three types of organic matter and one control) with three different times of incorporation. Each treatment was replicated six times. The results were subjected to ANOVA using a GLM model and the means were separated using Fisher's protected LSD test at the 0.05 probability level.

Results and discussion

The application of organic matter reduced the bulk density of the soil in relation to the control (Table 1). However, the impact of the different materials varied and the decline in bulk density was marginally greater when rice straw was applied, which could be attributed to the slower decomposition caused by the higher C:N ratio of this material. Incorporating *Tithonia* or rice straw 2 weeks before planting led to a lower bulk density, although the differences were non-significant. In contrast, the two green manures increased the CEC and organic carbon contents of the soil significantly. The highest increments in CEC were obtained for *Gliricidia* leaves applied 4 weeks before planting, followed by the application of *Tithonia* at the same time. As suggested by Kumar and Goh (2002), this could be attributed to the rapid decomposition of green manures when compared to rice straw. In contrast, the organic carbon content was highest after the application of rice straw, due to its slower decomposition rate and greater carbon content in relation to that of the two green manures.

Soil acidity was marginally increased by all the organic materials irrespective of the time of application, due to the release of organic acids. In contrast, green manures, especially *Gliricidia* leaves, increased soil N, while rice straw reduced it significantly compared to soils without organic matter. This reduction in soil N was due to nitrogen immobilization by the straw during early decomposition (Johnson et al., 2006). The greatest soil N content was recorded when *Gliricidia* leaves were added 2 weeks before planting. The reduction when this green manure was incorporated 4 weeks before planting could be due to leaching losses of the released N. The incorporation of *Tithonia*, which has a comparatively higher phosphorus content, increased this nutrient in the soil to a greater extent than the other organic materials, confirming earlier studies on upland soils by Cong and Merckx (2005). The increment in soil P was greater when the organic matter was incorporated 4 weeks before planting due to the slower release of this nutrient during decomposition when compared to N (Cobo et al., 2002). However, in overall terms, the application of organic matter, especially green manures, improved the soil properties, although the benefits varied with the material and time of incorporation.

Table 1

Impact of time of incorporation of organic matter on selected soil characteristics at the time of planting maize seeds

Organic matter	Time of incorp. (WBP)	BD	CEC	Org. C	pH	N	P
Gliricidia leaves	4	1.46	6.95	22.4	6.56	50.8	14.8
(C:N ratio 11.7)	2	1.49	6.42	23.5	6.62	55.7	14.1
Tithonia leaves	4	1.45	6.48	20.5	6.64	49.4	16.2
(C:N ratio 16.5)	2	1.43	6.12	21.6	6.69	48.5	15.9
Rice straw	4	1.41	6.20	24.6	6.74	42.6	13.4
(C:N ratio 49.1)	2	1.40	5.95	23.8	6.72	44.2	12.9
No organic matter$^+$		1.54	5.86	18.48	6.75	46.5	12.8
LSD$_{5\%}$ Time		0.12	0.41	1.42	NS	2.16	0.95
Org. matter		0.08	0.56	2.04	NS	1.95	0.48
Interaction		NS	*	*	NS	*	*

WBP: Weeks before planting; BD: Bulk density (g.cm^{-3}); CEC: Cation exchange capacity (m. eq. 100g^{-1}.soil); Org. C: Organic C (g. kg^{-1}); pH: 1:2. 5H$_2$O; N: mg g^{-1}; P: μg g^{-1}; $^+$: Control treatment; NS = Non-significant; * = Significant

The incorporation of all the organic materials at 2 or 4 weeks before planting induced the development of more roots (Table 2) in germinated maize seeds (V1 stage), compared to that in the control treatment. The incorporation of *Gliricidia* leaves at 2 or 4 weeks before planting did not cause significant differences in root growth at the V1 stage. However, the incorporation of *Tithonia* 4 weeks before planting induced a larger number of roots with greater length, due to the release of P (Table 1; Cong and Merckx, 2005) and to the beneficial impact of this nutrient on the root growth of maize (Sangakkara et al., 2004). The benefits in terms of root initiation and length were the lowest with rice straw, and the impact was greater when this crop residue was applied 4 weeks earlier, due to its slower decomposition. However, the incorporation of all the organic materials at the time of planting suppressed root number and length, compared to plants grown without additives. This could be due to the allelopathic effects of decomposing organic matter, which could affect root initiation and elongation, as discussed later.

The rates of root development over the V1–V4 growth stages (Table 3) illustrate the benefits of incorporating organic matter, especially green manures, to stimulate the growth of all types of roots of maize seedlings. *Tithonia* stimulated the growth of all roots, especially when added 4 weeks before planting, due to its ability to provide P for enhanced root growth. The beneficial impact of straw was also greater when added 4 weeks before planting. In contrast, root growth was promoted to the greatest extent by *Gliricidia* applied 2 weeks before planting, which could be attributed to the faster release of nutrients through the rapid decomposition caused by the lower C:N ratio, so if this material is added earlier, some nutrients such as N could be lost from the rhizosphere by leaching.

Table 2

Number and length (mm) of roots of maize seedlings 5 days after planting as affected by time of organic matter application

Organic matter	Time of incorp. (WBP)	No. of roots/seedling	Root length (mm)
Gliricidia leaves	4	15	245
	2	14	226
	0§	5	165
Tithonia leaves	4	18	395
	2	10	256
	0	6	142
Rice straw	4	11	219
	2	8	194
	0	4	125
No organic matter^{+}		7	196
$LSD_{5\%}$ Time		0.91	98.34
Organic matter		1.31	44.27
Interaction		*	*

WBP: Weeks before planting; $^{+}$: Control treatment; §: Application of organic matter at planting

Table 3

Rates of length development of different roots of maize seedlings as affected by time of organic matter incorporation (Calculations based on regression analysis)

Organic matter	Time of incorp.$^+$	Primary roots	Seminal roots	Nodal roots
Gliricidia leaves	2	$2149.5 \ln X + 1453.8$ (0.88)	$742.6 \ln X + 246.1$ (0.76)	$226.3 \ln X + 41.8$ (0.91)
	4	$2567.8 \ln X + 1066.7$ (0.95)	$925.8 \ln X + 425.7$ (0.95)	$315.4 \ln X - 38.1$ (0.71)
	0§	$1644.3 \ln X + 304.8$ (0.81)	$354.4 \ln X - 854.7$ (0.83)	$192.7 \ln X + 80.4$ (0.87)
Tithonia leaves	2	$2996.8 \ln X + 1109.4$ (0.91)	$1022.4 \ln X - 643.8$ (0.85)	$481.7 \ln X + 86.7$ (0.74)
	4	$2416.2 \ln X + 335.8$ (0.83)	$884.2 \ln X + 914.7$ (0.92)	$344.9 \ln X + 181.4$ (0.89)
	0	$1426.5 \ln X + 553.7$ (0.90)	$395.1 \ln X + 257.1$ (0.77)	$211.4 \ln X - 91.2$ (0.82)
Rice straw	2	$2042.7 \ln X + 446.8$ (0.93)	$661.8 \ln X + 255.9$ (0.73)	$185.3 \ln X + 26.7$ (0.80)
	4	$1826.4 \ln X + 1335.4$ (0.79)	$506.7 \ln X + 422.7$ (0.90)	$157.6 \ln X - 99.2$ (0.88)
	0	$1228.7 \ln X - 1044.8$ (0.85)	$318.9 \ln X + 366.7$ (0.83)	$126.5 \ln X + 49.3$ (0.77)
Control$^\#$		$1805.4 \ln X + 951.5$ (0.74)	$429.6 \ln X + 110.5$ (0.83)	$224.7 \ln X + 65.4$ (0.73)

$^+$: Weeks before planting; r^2 values are presented in parentheses; §: Application of organic matter at planting; $^\#$: No organic matter

The growth rates of all roots were significantly reduced when organic matter was added at the time of planting, as compared to that of seedlings grown in soil without the additives. The decomposition of organic matter releases phytotoxic compounds (Khanh et al., 2005). More importantly, Kamara et al. (2000), Tongma et al. (1998) and Chung et al. (2003) reported the allelopathic effects of *Gliricidia*, *Tithonia* leaves and rice straw, respectively, under laboratory and field conditions. The study on *Tithonia* reported the reduction of this effect after 5–7 days (Tongma et al., 1998). Hence, this phenomenon could be considered the causal factor in the suppression of the root growth of maize seedlings when the organic matter was added at planting, but further confirmatory experimentation on maize seedlings will be required. The incorporation of organic matter at 2 or 4 weeks before planting avoids these phytotoxic effects and promotes root growth in maize seedlings.

Root lengths measured at the V4 growth stage confirmed the impact of the selected organic matter and its time of incorporation on the root growth of maize seedlings (Table 4). The beneficial impact of *Gliricidia* leaves on the lengths of all roots was highest when added 2 weeks before planting, while that of *Tithonia* and straw was greatest when incorporated 4 weeks earlier. However, the lengths of all the roots at this stage were greater after the incorporation of *Tithonia* either 2 or 4 weeks before planting. This confirmed the stimulating effect of this green manure on the root development of maize seedlings, when added before planting. The addition of all three types of organic matter at planting reduced the root length compared to that of seedlings grown without additives. The adverse impact of the organic matter was most evident on the primary roots in the case of *Gliricidia* and *Tithonia* leaves, while straw did not cause similar reductions in the primary roots. The reduction in the length of primary roots of maize seedlings at the V4 stage due to *Tithonia* and *Gliricidia* leaves was 17% and 6%

when compared to that of seedlings grown without organic matter. This could be due to the early emergence of these roots in relation to others, thus subjecting them to phytotoxic effects. Although *Tithonia* stimulated root development when applied earlier, its application at planting retarded root development to the greatest extent. This phenomenon is important in developing a good root system for seedling establishment. Due to the slower decomposition associated with its greater C:N ratio, straw did not have a significant impact on the primary root growth of maize seedlings. In contrast, the adverse impact of incorporating straw at planting was greater on seminal and nodal roots, which emerge later. This suggests the later development of phytotoxic effects on the roots of maize seedlings from decomposing straw, and will require confirmatory studies.

The specific root lengths of maize seedlings were also affected by organic matter and the time of application (Table 4). The beneficial effects on this parameter of applying *Gliricidia* and *Tithonia* leaves or straw were observed when the material was added 2 or 4 weeks before planting. The specific root lengths were greatest when *Tithonia* was added, implying an expansion of the root system per unit weight when compared to plants supplied with *Gliricidia* or rice straw. Among the three organic materials used, rice straw induced the lowest specific root lengths, implying less stimulation of root expansion or a greater accumulation of dry matter. This will also require confirmatory studies under controlled conditions.

Table 4

Root length (mm) and specific root length (mm mg^{-1}) of maize at the V4 growth stage as affected by organic matter and time of incorporation

Organic matter	Time of incorporation^{+}	Root length			Specific root length		
		Prim. roots	Sem. roots	Nodal roots	Prim. roots	Sem. roots	Nodal roots
Gliricidia	4	2645	2495	526	102.5	128.7	94.2
leaves	2	2942	3222	642	125.4	146.3	115.4
	0§	1856	942	412	115..3	80.7	31.6
Tithonia	4	2945	3932	1349	182.1	156.7	121.6
leaves	2	2842	2585	784	169.7	131.5	102.1
	0	1642	995	582	96.1	75.8	24.5
Rice straw	4	2415	2584	480	95.4	85.3	66.7
	2	2085	1765	366	84.2	74.3	59.3
	0	1944	715	318	68.4	60.2	20.8
Control		1961	1187	516	124.5	96.1	46.2
LSD$_{5\%}$ Time		215.33	58.70	20.45	10.63	4.56	5.01
Org.matter		401.88	61.37	55.05	14.30	6.14	3.94
Interaction		NS	*	*	NS	*	*

$^{+}$: Weeks before planting; *: Significant; NS: Non-significant; §: Application of organic matter at planting

The branching index at the V4 stage (Table 5) confirmed the ability of organic matter to promote root expansion and branching. Incorporating *Tithonia* leaves 4 weeks before planting produced the highest branching index among all the treatments, followed by the addition of *Gliricidia* leaves 2 weeks before planting. For straw, the highest branching index was recorded when it was applied 4 weeks before planting. This again confirmed the benefits of green manures in stimulating root growth, although the best time of application varied. As in root development, the branching index was suppressed by the incorporation of organic matter at the time of planting, indicating the existence of allelopathic effects on root branching as well as on initiation and growth.

Table 5

Branching index of maize roots at the V4 growth stage as influenced by organic matter and time of incorporation

Organic matter	Time of incorporation (WBP)	Branching index*
Gliricidia leaves	4	1.14 ± 0.11
	2	1.31 ± 0.08
	0§	0.72 ± 0.22
Tithonia leaves	4	1.79 ± 0.19
	2	1.18 ± 0.05
	0	0.96 ± 0.04
Rice straw	4	1.26 ± 0.20
	2	1.02 ± 0.06
	0	0.53 ± 0.31
No organic matter⁺		0.86 ± 0.19

Data are means of 5 replicates ± standard errors; WBP: Weeks before planting; Branching index: Length of secondary and nodal roots/length of primary root (adapted from Tsuji et al., 2005); §: Application of organic matter at planting; ⁺: Control treatment

Conclusions

Tropical smallholders cultivate maize under rainfed marginal conditions, using organic matter to either supplement chemical fertilizers or as alternative sources to provide nutrients. The material used ranges from crop residues, such as rice straw, to green manures obtained from the surroundings, depending on availability, labour and time. The judicious management of the applied organic matter, especially application at the correct time, is vital for obtaining the desired results, especially in terms of better crop growth during early establishment, which is affected by the development of a good root system. This study highlights the fact that the time of application has a significant impact on the root growth of maize seedlings. The application of organic matter such as *Gliricidia* (legume) leaves with a low C:N ratio at two weeks before planting induced the best root development in maize seedlings. In contrast, materials such as *Tithonia* leaves or rice straw, which have higher C:N ratios, are best applied earlier (i.e. 4 weeks before planting) if optimal root growth is to be ensured. The application of all types of plant-based organic matter at the time of maize planting should be avoided, as it retards the development of the roots, possibly due to allelopathic effects. These results could have a bearing on organic farming systems, which are becoming popular in the tropical regions.

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INVESTIGATION ON DIRECT AND RECIPROCAL CROSSES IN MAIZE (*Zea mays* L.)

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Seven traits of twelve hybrids derived from direct and reciprocal crosses of four parental lines were examined during three years in Hungary. No significant differences were found between the direct and reciprocal crosses for stalk diameter or leaf number over the average of three years in any of the hybrids. Significant differences in the number of primary tassel branches were observed between UDH5 and its reciprocal UDH8, and between UDH6 and its reciprocal UDH11. It was evident in both instances that the degree of heterosis in the reciprocal crosses far exceeded that in the direct ones. A high number of tassel branches was dominant over a low number, so this trait was preferentially inherited in the hybrids. A positive correlation was observed between plant height and main ear attachment height ($r = 0.67^{**}$). A positive correlation ($r = 0.89^{**}$) was also found between the length of the main tassel axis above the lowest and above the uppermost side-branch. A medium correlation was observed between the number of primary tassel branches and the plant height ($r = -0.42^{**}$). The number of primary tassel branches exhibited the highest heterosis. These results can be utilized in practical selection and seed production.

Key words: direct and reciprocal crosses, heterosis, maize (*Zea mays* L.), plant height, ear attachment height, tassel characteristics

Introduction

In maize selection, it is of great importance to know the performance and combining ability of the crossing partners. The knowledge of the direction of the reciprocity effects for both characters is only possible if direct and reciprocal hybrids are analysed. A number of Hungarian authors draw attention to this field (Berzy et al., 2005). Kovács (1963) investigated direct and reciprocal single crosses derived from Martonvásár lines and found no difference between the productivity of the two hybrids.

Nagy (1982) studied the days until 75% tassel flowering, the seed moisture content % at harvest and the dry kernel yield/plant (g) in crosses of lines with different ripening times, and found a significant cytoplasmic effect in the case of one line in relation to dry kernel yield/plant. It was concluded that the maternal effect could be detected not later than flowering. Nagy (1985) examined the leaf number, ear attachment height, ear length, cob weight, number of kernel rows, ear weight, kernel weight, 500-kernel weight, shelling % and oil content in 14 early SC hybrids and their reciprocal crosses. In a quarter of the data pairs compared, a significant difference was found at $P \geq 5\%$. In the case of leaf number and cob weight, the male crossing partner determined the value of the F_1 generation with higher frequency, but for most features (ear attachment height, ear length, number of kernel rows, ear weight, kernel weight, 500-kernel weight) a maternal effect was experienced.

Gyenesné Hegyi et al. (2001) studied the protein and oil contents of 12 direct and 12 reciprocal hybrids and found no heterosis compared to the parental mean. In ten cases there was a significant difference in protein content, while no statistical difference was found in the oil content between direct and reciprocal hybrids.

Plant height and the attachment height of the main ear were examined by several authors (Gyenesné Hegyi et al., 2002a, b; Hegyi et al., 2005a, b; Zsubori et al., 2002), who concluded that these were among the most important variety trait pairs and were closely correlated to other characteristics.

Singh (1965) reported significant reciprocal differences between ear attachment height and plant height, while Bonea and Urechean (2003) studied the relationship between grain yield and crude protein content percentage in 15 direct and reciprocal crosses and found significant differences between the direct and reciprocal hybrids. Schuetz and Mock (1978) examined the inheritance of tassel branch number in six crosses and detected additive, dominant and epistatic gene effects, of which the additive effects were the most important.

Khehra and Bhalla (1976) crossed ten genetically different maize lines, including reciprocals. Larger differences were observed with respect to the reciprocal effect in early × late variety combinations than in early × early or late × late ones. Few data are available in the literature on tassel characteristics in direct and reciprocal crosses.

Materials and methods

Field trials were set up on the experimental area of the Genetics Team (Centre of Agricultural Sciences, University of Debrecen, Hungary) between 2004 and 2006, to study twelve maize hybrids (UDH1, 2, 3, 5, 6, 9 direct and UDH4, 7, 8, 10, 11, 12, reciprocal crosses) produced from four irradiated inbred lines (UDL1, UDL4, UDL5, UDL6) (Table 1). The experimental procedures were reported by Bódi et al. (2006). The soil was leached chernozem with a non-calcareous upper layer. The subsoil was 7–9 m below the surface. The humus layer had a medium depth of 50–70 cm, with an organic matter content of 2.57%. N, P_2O_5 and K_2O fertilizers were applied in doses of 100, 90 and 90 kg ha^{-1}, respectively. Phosphorus and potassium were applied in autumn, while 30% of the nitrogen was applied in autumn and 70% in spring. Each plot consisted of two 5-m rows, with 70 cm between rows and a plant-to-plant distance of 20 cm, giving 50 plants/plot. The weather in 2004–2006 provided optimal conditions for the vegetative development and grain yield production of maize. Table 1 shows the genotypic composition of the complete diallel system. Characteristics were averaged over 15 plants/plot, following the CPVO TP/2/2 guidelines (Tables 2, 5 and 6). The data were processed by analysis of variance and correlation analysis using SPSS 13 for Windows.

Table 1
Genetic composition of the maize hybrids investigated

Lines	UDL1	UDL4	UDL5	UDL6
UDL1	–	UDH1	UDH2	UDH3
UDL4	UDH4	–	UDH5	UDH6
UDL5	UDH7	UDH8	–	UDH9
UDL6	UDH10	UDH11	UDH12	–

Table 2
Morphological features of the inbred lines included in the experiment (averaged over 2004–2006)

Line	Plant height (cm)	Attachment height of main ear (cm)	No. of primary tassel branches	Tassel length[1] (cm)	Tassel length[2] (cm)	Stalk diameter (mm)	No. of leaves
UDL1	174.8	65.6	1.4	23.4	19.7	19.6	11.4
UDL4	163.9	51.7	6.9	28.3	20.3	19.0	10.7
UDL5	176.8	53.5	6.6	34.0	26.4	17.1	10.5
UDL6	160.5	50.8	9.1	33.8	25.7	15.1	11.1

[1]: Length of main tassel axis above lowest side branch; [2]: Length of main tassel axis above uppermost side branch

Results and discussion

The mean square (MS) values indicate that each character tested was influenced to the greatest extent by the genotype, followed by the year (Tables 3 and 4). Analysis of variance indicated that the genotype influenced all traits at the 1% level of significance, with the exception of stalk diameter (P=5%). The MS values for the year were also significant at the 1% level except for stalk diameter.

The genotype × year interaction was significant at the 0.1% level for plant height, the attachment height of the main ear and the number of primary branches. This contradicted the findings of Russell (1976), who indicated no significant genotype × year interaction for plant height or ear attachment height. The genotype × year interaction for the length of the main tassel axis above the lowest side-branch was significant at 5%, while those for the length of the main tassel axis above the uppermost branch, for stalk diameter and for leaf number were not.

Table 3
Analysis of variance for plant characters

Source of variance	FG	Plant height		Attachment height[+]		Stalk diameter		No. of leaves	
		MS	F value	MS	F value	MS	F value	MS	F value
Genotype	11	4696.66	80.51**	1157.94	31.30**	13.00	3.28*	3.78	7.95**
Year	2	4762.31	81.63**	629.19	18.71**	14.90	3.76^{ns}	13.83	29.11**
Genotype × year	22	403.02	6.90**	141.69	3.83**	3.02	0.76^{ns}	0.75	1.57^{ns}
Error	72	58.33		36.99		3.96		0.47	

[+]: of main ear; *, **: Significant at the P = 5 % and P = 1% level, respectively; ns: non-significant

Table 4
Analysis of variance for tassel characteristics

Source of variance	FG	No. of primary branches		Tassel length^1		Tassel length^2	
		MS	F value	MS	F value	MS	F value
Genotype	11	38.14	17.75**	16.92	5.26**	16.02	3.68**
Year	2	49.55	23.07**	494.93	154.02**	323.37	74.32**
Genotype × year	22	7.51	3.49**	7.63	2.37*	7.00	1.60^{NS}
Error	72	2.14		4.35		3.96	

*, **: Significant at the P = 5 % and P = 1% level, respectively; NS: non-significant; ^{1}: Length of main tassel axis above lowest side branch; ^{2}: Length of main tassel axis above uppermost side branch

Differences between the hybrids in morphological features were only significant in a few cases (Table 5). Among the factors examined, genotype had the greatest effect on plant height, which varied between 198.7 cm (UDH11) and 252.7 (UDH3), averaged over the three years (Table 5). The second tallest hybrid was UDH2 (250.8 cm), and the ear attachment height of UDH3 and UDH2 was also the greatest (90.4 and 88.4 cm, respectively) on average. The maternal components of these hybrids (UDL1 and UDL5) were the tallest of the lines (Table 2), while UDL1 and UDL6 had the largest number of leaves (Table 2). The hybrids with the largest leaf numbers (UDH2 and UDH7) were obtained from crosses involving UDL1. Statistically significant differences between direct and reciprocal crosses were found only in a few instances in the case of plant height and ear attachment height (Table 5).

The results confirm those of Gyenes-Hegyi et al. (2002a), who found that significant differences between direct and reciprocal crosses were not of importance for research, as the differences were smaller than those needed to distinguish between two hybrids (based on CPVO TP2/2 guidelines). In the case of stalk diameter and leaf number there were slight, statistically insignificant changes.

Table 5
Morphological characteristics of the hybrids investigated

Hybrids	Plant height (cm)	Degree of heterosis (%)	Ear attachment height (cm)	Degree of heterosis (%)	Stalk diameter (mm)	Degree of heterosis (%)	No. of leaves	Degree of heterosis (%)
UDH1	238.3^{NS}	+40.7	80.2^{NS}	+36.7	22.2^{NS}	+15.2	12.2^{NS}	+10.7
UDH4	241.7^{NS}	+42.7	77.7^{NS}	+30.5	21.6^{NS}	+17.9	12.4^{NS}	+11.8
UDH2	250.8^{NS}	+42.6	88.4^{NS}	+48.2	21.4^{NS}	+17.0	13.4^{NS}	+22.7
UDH7	243.5^{NS}	+38.6	83.7^{NS}	+40.4	20.6^{NS}	+12.2	13.3^{NS}	+21.5
UDH3	252.7^{NS}	+50.7	90.4^{NS}	+55.4	23.1^{NS}	+33.1	12.4^{NS}	+12.2
UDH10	238.7^{NS}	+42.4	88.4^{NS}	+48.9	21.4^{NS}	+23.3	13.1^{NS}	+16.0
UDH5	208.8*	+20.2	57.2^{NS}	+8.7	21.0^{NS}	+16.4	11.3^{NS}	+6.4
UDH8	214.1*	+25.6	62.4^{NS}	+18.7	20.9^{NS}	+16.1	11.7^{NS}	+10.5
UDH6	202.8^{NS}	+25.1	59.6*	+16.2	19.9^{NS}	+17.0	11.9^{NS}	+8.7
UDH11	198.7^{NS}	+22.5	67.1*	+30.7	19.6^{NS}	+15.1	12.1^{NS}	+10.8
UDH9	207.1*	+22.8	72.1*	+38.3	20.6^{NS}	+28.0	12.0^{NS}	+11.2
UDH12	202.2*	+19.2	65.2*	+29.7	18.5^{NS}	+15.4	12.1^{NS}	+11.3

*Significant at the P = 5% level; ^{NS} Non-significant

Statistically significant differences for the number of primary tassel branches were found between the reciprocal pairs UDH5–UDH8 and UDH6–UDH11 (Table 6). In both cases, the degree of heterosis in the reciprocal crosses far exceeded that in the direct crosses. Averaged over three years, UDL1 had 1.4 primary tassel branches, while UDL6 had 9.1 (Table 2). UDL6 increased the number of tassel branches in the hybrids irrespective of whether it was male or female crossing partner. This is in agreement with the findings of Mock and Schuetz (1974) and Gyenesné Hegyi et al. (2002c). With respect to tassel length, a significant difference was only recorded between UDH3 and UDH10. The hybrids exhibited 23–30% heterosis compared to the mean for the parental lines. A greater range of heterosis (10.7–42.8%) was found for the length of the main tassel axis above the uppermost side-branch. With the exception of UDH5 and UDH8, the direct crosses showed a larger hybrid effect for this trait.

To determine the interactions more precisely, correlations were calculated between the examined traits (Table 7). There was a strong positive correlation between plant height and main ear attachment height, as also observed by Gyenes-Hegyi et al. (2002a). There was also a strong positive correlation (0.89) between the length of the main tassel axis above the lowest and above the uppermost side branch. A medium but significant negative correlation was observed between plant height (–0.42**) and the number of primary tassel branches, and between the main ear attachment height and the number of primary tassel branches (–0.31**). Stalk diameter was significantly correlated with the plant height and the main ear attachment height, and negatively correlated with the number of primary tassel branches. A medium but significant correlation was found between leaf number and plant height (0.52**) and between leaf number and the main ear attachment height (0.63**), while there was only a loose correlation between leaf number and the two tassel characteristics.

Table 6

Tassel characteristics of investigated hybrids

Hybrids	Branches (1) (cm)	Degree of heterosis (%)	Tassel lenght (2) (cm)	Degree of heterosis (%)	Tassel lenght (3) (cm)	Degree of heterosis (%)
UDH1	6.5^{NS}	+56.1	36.9^{NS}	+28.6	28.6^{NS}	+42.8
UDH4	7.6^{NS}	+81.4	35.6^{NS}	+29.6	29.8^{NS}	+35.6
UDH2	4.6^{NS}	+14.2	37.4^{NS}	+30.8	30.8^{NS}	+29.0
UDH7	5.1^{NS}	+26.0	36.4^{NS}	+27.1	27.1^{NS}	+24.7
UDH3	6.8^{NS}	+28.7	39.5*	+23.1	23.1*	+35.6
UDH10	6.0^{NS}	+13.8	35.4*	+26.8	26.8*	+18.0
UDH5	7.6*	+11.5	33.7^{NS}	+26.5	26.5^{NS}	+13.1
UDH8	9.7*	+42.9	37.2^{NS}	+29.3	29.3^{NS}	+25.0
UDH6	9.4*	+17.6	35.6^{NS}	+27.4	27.4^{NS}	+19.1
UDH11	11.9*	+48.4	36.9^{NS}	+27.1	27.1^{NS}	+17.7
UDH9	8.2^{NS}	+3.9	36.8^{NS}	+29.0	29.0^{NS}	+11.2
UDH12	7.4^{NS}	–6.3	36.3^{NS}	+28.8	28.8^{NS}	+10.7

1: Number of primary tassel branches; 2: Length of main tassel axis above lowest side branch; 3: Length of main tassel axis above uppermost side-branch; *: Significant at the P = 5% level, ^{NS}: Non-significant

Table 7
Correlations between investigated characteristics

	1	2	3	4	5	6	7
1	1.00						
2	0.67**	1.00					
3	–0.42**	–0.31**	1.00				
4	0.34^{NS}	0.38**	0.32**	1.00			
5	0.02^{NS}	0.33^{NS}	0.10^{NS}	0.89**	1.00		
6	0.31**	0.22**	–0.26**	0.18^{NS}	0.26^{NS}	1.00	
7	0.52**	0.63**	–0.14^{ns}	0.32**	0.30**	0.40^{NS}	1.00

1: Plant height; 2: Attachment height of main ear; 3: Tassel: Number of primary branches; 4: Tassel: length of main axis above lowest side-branch; 5: Tassel: length of main axis above uppermost side-branch; 6: Stalk diameter; 7: Number of leaves; **: Correlation significant at the P = 1% level; ^{NS} Non-significant

Averaged over three years, statistically significant differences between direct and reciprocal crosses were only observed in a few instances. Heterosis for tassel branch number was of great importance, since the tassel branch number plays a very significant role in the pollen-producing ability of both the parent and the hybrid (Bódi and Pepó, 2007), and should therefore be given due consideration during the production of hybrid combinations. For this trait, the largest degree of heterosis was observed in crosses involving the line with the largest number of tassel branches, regardless of whether it was the male or female partner. These results can be utilized in practical selection and seed production.

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IMPACT OF DISTILLERY EFFLUENT APPLICATION ALONG WITH INORGANIC FERTILIZERS ON DRY MATTER YIELD AND MINERAL COMPOSITION OF RICE

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A greenhouse experiment was conducted to study the impact of premethanation (PREME) and postmethanation (POME) distillery effluent applied as pre-sowing irrigation (PSI) along with graded levels of inorganic fertilizers on the grain and straw yield and nutrient content of a rice crop (var. PR 116). Maximum grain yield (29.4 g pot^{-1}) was recorded with the application of 100% recommended NPK along with one pre-sowing irrigation (PSI) through POME and the lowest yield (7.4 g pot^{-1}) was obtained with 2 PSI applied through PREME without any inorganic fertilizers. The application of POME equivalent to 1 PSI was more effective in increasing the grain and straw yield of rice than no POME application or POME application equivalent to 2 PSI. A significant decrease in yield occurred with the application of 2 PSI of either effluent, and beyond 2 PSI the rice seedlings did not grow.

In comparison to POME, the application of PREME increased the content of K (5%), Cu (10%), Fe (17%) and Mo (21%), but decreased that of P (12%), S (5%) and Mg (11%) in rice grain. In rice straw an increase was only observed in K (9%) and Mo (14%), while the contents of other nutrients (P, S, Zn, Cu, Mn) decreased by 8 to 21%. An increase in the level of effluent from 0 to 2 PSI significantly increased the content of N (by 21%), K (11%), S (10%), Zn (9%), Cu (21%) and Mo (8%), but decreased that of P (16%) and Mg (19%) in rice grain. In the case of rice straw, an increase in K (28%), S (32%), Cu (65%) and Mo (45%) content was recorded. Effluent application, inorganic fertilizers and their interactions had a significant effect on both the grain and straw yields and on the nutrient concentration in the plants.

Key words: distillery effluent, inorganic fertilizer, dry matter yield, mineral content, rice

Introduction

Alcohol (ethanol) is mainly produced in India by the fermentation of diluted sugarcane molasses solution. After the separation of alcohol by distillation, the large volumes of residual liquid (alcohol : waste liquid :: 1 : 15) discharged as waste water is known as distillery effluent or spentwash. It is

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characterized by high biochemical and chemical oxygen demand (50,000 mg l^{-1} BOD and 95,000 mg l^{-1} COD), high salt content (≈ 15 dS m^{-1} electrical conductivity) and acidic pH (Chhonkar et al., 2000). Most of the effluent is disposed of by dumping it on a waste land, which is unable to assimilate such a large quantity. Due to the high organic load, stagnating effluent at low-lying sites acts as a great source of environmental pollution, leading to a number of health hazards. Treatment of the effluent in biomethanation digesters leads to a significant reduction in its organic load. Treated distillery effluent had 3 to 5 times lower biochemical oxygen demand (BOD) and chemical oxygen demand (COD) values, fairly high contents of N, P, K, Ca, Mg, Cl and S, appreciable amounts of essential micronutrients and alkaline pH (Nagappan et al., 1996). Instead of the haphazard disposal of the effluent on waste land, the distillery effluent can be used for irrigation purposes without any significant adverse impact on crop growth provided it is diluted to a BOD level of below 1,000 mg l^{-1} (Pathak et al., 1998). The application of a 6 cm depth of postmethanation effluent (1,000 mg l^{-1} BOD) would add about 160 kg N, 2100 kg K, 11 kg P and 540 kg S ha^{-1} to the soil, thus reducing the nutrient requirement through fertilizers (Joshi et al., 1996).

The continuous use of large quantities of chemical fertilizers in intensive cropping systems results in various problems and unfavourable soil conditions (Parr et al., 1986). Among the means available to achieve sustainability in agricultural production, organic matter plays a key role. The utilization of distillery wastewater rich in organic matter for the irrigation of crops may provide a satisfactory solution. In Punjab, there are eight distilleries generating 1317 million litres of effluent a year (Joshi, 1999). Thus, there is a need to develop a suitable methodology for the utilization of distillery effluent to supplement essential nutrients for crop growth. The present investigation was undertaken to study the effect of distillery effluents, both premethanation (PREME) and postmethanation (POME), when applied along with inorganic fertilizers, on the dry matter yield and mineral composition of rice.

Materials and methods

Distillery effluents were collected in bulk before and after methanation from the waste disposal point of a molasses-based distillery producing alcoholic beverages, situated along the grand trunk road at Hamira, Distt Jallandhar, Punjab, India. This facility produces 14 million cases of potable alcohol a year and is ranked as the largest distillery of its kind in Asia. Three samples, each consisting of 20–25 l, were collected at weekly intervals and mixed together thoroughly before use in the experiment. A greenhouse experiment was conducted by growing rice (var. PR 116) in polythene-lined earthen pots containing 4 kg of alkaline (pH 8.3) loamy sand soil. The treatments consisted of two types of effluent, premethanation (PREME) and postmethanation (POME), each applied at five levels (0, 0.5, 1, 2 and 3 l pot^{-1}) equivalent to 0, 1, 2, 4 and 6 pre-sowing irrigations (PSI), and five levels of inorganic fertilizers (0, 25, 50, 75 and 100% of the recommended NPK level of 120 kg N, 30 kg P_2O_5 and 30 kg K_2O ha^{-1}) in four replications. The 100% recommended NPK level was achieved by adding 48 mg N, 12 mg P_2O_5 and 12 mg K_2O

kg^{-1} soil. A single application of effluent was made 20 days before transplanting the rice seedlings to the respective pots. All the subsequent irrigations were applied using fresh tubewell water only. One-third of N along with the full dose of P and K was applied before transplanting and the rest of the N was applied in two equal splits, 3 and 6 weeks after transplanting. Initially six rice seedlings were transplanted to each pot, thinned to four when they were well established. The crop was harvested at maturity. Samples of straw and grain were washed free of any surface contamination and dried to constant weight in an oven at 60±5°C, after which the dry matter yield was recorded.

The chemical characteristics of the distillery effluents were determined using standard procedures. The biochemical oxygen demand (BOD) and chemical oxygen demand (COD) of the effluent were determined as described by Eaton et al. (1995). Samples of effluent, straw and grain were digested in concentrated nitric acid followed by perchloric acid treatment and the digest was analysed for various elements (except N) using an ICAP spectrophotometer (GBC, Australia). For the estimation of nitrogen, plant and effluent samples were digested in concentrated H_2SO_4 containing 1.9% SeO_2 and N was determined using an auto-analyser (Technicon). Data pertaining to the dry matter yield and nutrient composition were statistically analysed for a completely randomized design as described by Singh et al. (1984).

Results and discussion

Considerable differences were observed in the nature and elemental composition of the two effluents, premethanation (PREME) and postmethanation (POME) (Table 1). In comparison to PREME, POME was alkaline in reaction and had 3 to 4 times lower total solids and BOD and COD values. There was also a reduction of 1.3–4.0 times for essential primary nutrients, 9–17 times for secondary nutrients, 0–22 times for micronutrients and 0–10 times for toxic heavy metals. The chemical characteristics of the distillery effluents observed in the present investigation are comparable to those reported by Castello-Branco et al. (1988), Joshi et al. (1996), Rajukkannu and Manickam (1997) and Chhonkar et al. (2000). The contents of heavy metals (Cd and Cr) compare well with those reported by Joshi (1999). The suitability of distillery effluents as a substitute for conventional fertilizers depends mainly on their BOD and COD values. Since postmethanation effluent (POME) has 3–4 times lower BOD and COD and greater pH values (8.42) than PREME (pH 5.63) and contains a sufficient quantity of essential nutrients, it could be more suitable for agricultural purposes.

Grain and straw yield of rice

Irrespective of the type of effluent and the amount of inorganic fertilizer applied, rice seedlings were unable to grow in pots receiving distillery effluent equivalent to four or six pre-sowing irrigations (PSI). The utilization of distillery effluent with high BOD, COD and total dissolved salts could prove detrimental to crop growth by causing anaerobic conditions, increasing pCO_2 and producing organic acids during its decomposition. An appreciable increase in total dissolved solids (TDS) in piezometric samples ranging from 441.6 to 998.4 mg l^{-1} has been observed after the application of postmethanation distillery effluent at 225 m^3 ha^{-1} (112.5 ml kg^{-1} soil) for two years as pre-sowing irrigation to a field under a rice-wheat sequence (Jain et al., 2005). The content of Ca, Mg, Na, K, Cl and SO_4 ions increased 3–4 times in the soil leachate after a single pre-sowing irrigation with spentwash at 500 m^3 ha^{-1} (250 ml kg^{-1} soil). When using distillery effluent having a BOD value of 3200 mg/l for rice, Sahai et al. (1983) observed that only a 5% concentration was beneficial for rice. In a field experiment, Chhonkar et al. (2000) observed discernable toxic effects on maize, wheat and rice crops irrigated with a 40% concentration of postmethanated distillery effluent. In the present investigation, the electrical conductivity also increased 3–4 times after the application of four presowing irrigations and the increase was greater in the case of PREME. Thus, greater salt content coupled with high BOD and COD may be responsible for crop failure in the case of four or six presowing irrigations with distillery effluent.

Table 1

Chemical characteristics of the distillery effluent

Parameters	Premethanation effluent (PREME) (Mean ± SD), n=3	Postmethanation effluent (POME) (Mean ± SD), n=3
pH	5.6 ± 0.04	8.4 ± 0.02
Electrical conductivity, dS m^{-1}	18.1 ± 0.24	17.6 ± 0.14
Organic carbon, %	0.82 ± 0.02	0.54 ± 0.02
Total solids (g l^{-1})	60.4 ± 0.62	21.3 ± 0.02
Biological oxygen demand (BOD), mg l^{-1}	36,300 (n=2)	11,150 (n=2)
Chemical oxygen demand (COD), mg l^{-1}	51,920 (n=2)	11,520 (n=2)
Macronutrients, mg l^{-1}		
N	1275 ± 25	337 ± 37.5
P	791 ± 177	396 ± 129
K	6947 ± 117	5152 ± 158
S	3147 ± 85	369 ± 12
Ca	1234 ± 37	80.9 ± 16
Mg	1424 ± 43	847 ± 26
Na	491 ± 54	326 ± 49
Micronutrients, mg l^{-1}		
Mn	9.6 ± 0.6	1.5 ± 0.3
Zn	3.2 ± 0.8	3.5 ± 1.4
Fe	1516 ± 149	71 ± 9
Cu	0.3 ± 0.2	0.2 ± 0.1
Mo	1.2 ± 0.3	0.9 ± 0.3
B	8.0 ± 2.1	3.2 ± 3.5
Heavy metals, mg l^{-1}		
Cd	0.22 ± 0.04	0.02 ± 0.01
Cr	0.90 ± 0.10	0.32 ± 0.10
Ni	0.45 ± 0.05	0.21 ± 0.02
Pb	1.37 ± 0.20	0.46 ± 0.20

With the application of different combinations of distillery effluent and inorganic fertilizer, the grain and straw yield of rice varied from 7.5 to 29.4 and 9.2 to 37.2 g pot^{-1}, respectively (Table 2). Maximum dry matter yield was obtained for both grain and straw with the application of POME equivalent to 1 PSI along with 100% of recommended fertilizers, whereas the minimum yield was recorded for 2 PSI applied with PREME without inorganic fertilizer. Significantly lower dry matter yield was recorded after the application of PREME as compared to that of POME. The decrease in the dry matter yield of rice due to the application of PREME could be attributed to its relatively higher BOD and COD values as compared to POME (Table 1). Lower rice yields due to the application of effluent having a BOD value of over 1500 mg l^{-1} was also reported by Pathak et al. (1998). The positive influence of postmethanation distillery effluent (up to 30% concentration) on rice crops were observed by Chhonkar et al. (2000).

The application of POME equivalent to one pre-sowing irrigation (1 PSI) resulted in a significant increase in the grain and straw yield of rice, but in the case of PREME the yield remained statistically at par with the control. Compared to 1 PSI, a decrease in grain yield was recorded for both types of

Table 2

Effect of pre-sowing irrigation with distillery effluent and inorganic fertilizer levels on dry matter yield of rice

Effluent levels	Inorganic fertilizer levels (% of recommended NPK)											
	0	25	50	75	100	Mean	0	25	50	75	100	Mean
	Postmethanation effluent (POME)						Premethanation effluent (PREME)					
	Grain yield, g pot^{-1}											
0*	9.9	11.0	13.0	14.7	15.9	12.9	9.9	11.0	13.0	14.7	15.9	12.9
1 PSI*	11.4	10.9	22.2	23.4	29.4	19.5	12.7	14.0	14.1	13.2	11.7	13.1
2 PSI*	13.5	17.0	18.8	16.3	17.1	16.5	7.6	7.5	8.4	8.1	8.6	8.0
Mean	11.6	13.0	18.0	18.1	20.8	16.3	10.1	10.8	11.8	12.0	12.1	11.4
	Straw yield, g pot^{-1}											
0	14.2	13.7	18.3	19.6	20.3	17.2	14.2	13.7	18.3	19.6	20.3	17.2
1 PSI	16.1	13.5	29.7	33.2	37.2	25.9	17.9	17.6	19.8	16.6	15.6	17.5
2 PSI	16.1	24.7	25.5	22.7	21.8	22.1	9.2	10.9	10.2	11.7	11.5	10.7
Mean	15.5	17.3	24.5	25.2	26.4	21.8	13.8	14.1	16.1	16.0	15.8	15.1

LSD ($P<0.05$)	Grain	Straw
Type of effluent	0.56	0.74
Levels of effluent	0.68	0.91
Interaction between type and level of effluent	0.88	1.17
Overall mean of inorganic fertilizer levels	0.96	1.29
Interaction between type of effluent and inorganic fertilizer levels	1.24	1.66
Interaction between levels of effluent and inorganic fertilizer levels	1.52	2.03
Overall interaction	2.15	2.88

PSI: Pre-sowing irrigation; Recommended NPK rate (mg kg^{-1} soil): 48–12–12 effluents applied at 2 PSI. However, the decrease was greater for PREME (39%) than for POME (15%). This decrease in dry matter yield could be attributed to the relatively higher BOD and COD values of PREME as compared to those of POME. In comparison to the control, pre-sowing irrigation with spentwash at 125 m^3 ha^{-1} (62.5 ml kg^{-1} soil) increased the grain yield of rice by 1.7 times under field conditions (Mahimairaja and Bolan, 2004). A further increase in the rate of spentwash to 250 or 500 m^3 ha^{-1}, however, proved detrimental to crop growth and the grain yield decreased 0.74 and 0.56 times, respectively. Regular irrigation with diluted effluent has also proved useful to crop growth. In a field experiment, Annadurai et al. (1999) observed the lowest grain yield (1.3 t ha^{-1}) when rice was irrigated with 10 times diluted effluents only. As the dilution of the distillery effluents increased to 50 times, the grain yield increased to 4.1 t ha^{-1}. The highest grain yield of maize (3.69 t ha^{-1}) was obtained with the application of biomethanated spentwash, followed by raw spentwash (3.22 t ha^{-1}) (Ramana et al., 2002).

The application of inorganic fertilizer proved more beneficial to the rice crop in the presence of POME than for PREME (Table 2). In the case of POME, a significant increase in the grain and straw yield was observed even with the application of 25% of recommended NPK and it continued to increase significantly with a further increase in the level of inorganic fertilizer, the maximum grain and straw yield being recorded at 100% of recommended NPK. In the case of PREME, a significant increase in yield was only observed at 50% of recommended fertilizer application and a further increase in the amount of fertilizer applied had no beneficial effect. Similar results were reported by Zalawadia and Ramana (1994) with the application of different levels of NPK along with distillery waste to sorghum. Supplementation with inorganic fertilizers (NPK) helps in achieving the full manuring potential of the distillery effluent (Ramana et al., 2002).

Mineral composition of rice grain and straw

Significant changes were observed in all the major and minor nutrients except for Mn and B in rice grain (Tables 3–5) and N, Fe and B in rice straw (Tables 6–8). The nitrogen content of the grain and straw varied from 0.67 to 1.36 % and 0.23 to 0.54%, respectively, under different combinations of effluent and inorganic fertilizer (Tables 3, 6). In general, the application of either POME or PREME did not show any significant effect on the mean N content of either rice grain or straw. When the level of effluent increased from 0 to 2 PSI, only the N content of the grain increased significantly from 0.87 to 1.04% for POME and from 0.87 to 1.07% for PREME. Compared to the control, the N content of the grain increased significantly after the application of inorganic fertilizer at 25% of the recommended level. The N content increased significantly with a further increase in the level of fertilizer to 50% of the recommended dose. However, an increase in the fertilizer level to 75 or 100% of the recommended dose did not increase the N content compared with 50% of recommended NPK.

Table 3

Effect of pre-sowing irrigation with distillery effluent and inorganic fertilizer levels on the macronutrient content of rice grain

Effluent levels	Inorganic fertilizer levels (% of recommended NPK)											
	0	25	50	75	100	Mean	0	25	50	75	100	Mean
	Postmethanation effluent (POME)						Premethanation effluent (PREME)					
	Nitrogen (%)											
0	0.75	0.78	0.90	0.93	0.98	0.87	0.75	0.78	0.90	0.93	0.98	0.87
1 PSI	0.67	0.71	1.23	1.00	1.28	0.98	1.06	1.18	1.01	0.94	0.74	0.99
2 PSI	0.82	1.36	1.01	1.08	0.94	1.04	0.97	1.04	1.08	0.97	1.31	1.07
Mean	0.75	0.95	1.05	1.00	1.07	0.96	0.93	1.00	1.00	0.95	1.01	0.98
	Phosphorus (mg kg^{-1})											
0	2758	4358	3941	4254	4206	3903	2758	4358	3941	4254	4206	3903
1 PSI	3377	3395	3508	4045	4129	3691	2928	4147	3845	3787	3669	3671
2 PSI	4071	3787	4003	4163	4004	4006	3007	2483	2767	2291	2402	2590
Mean	3402	3847	3817	4154	4113	3867	2898	3663	3517	3444	3426	3388
	Potassium (mg kg^{-1})											
0	4201	4490	3755	3791	4080	4063	4201	4490	3755	3791	4080	4063
1 PSI	4140	3791	4237	4410	4583	4232	4698	4089	4376	4196	4250	4322
2 PSI	4362	4197	4231	4181	4261	4246	4836	4576	4983	4373	5160	4786
Mean	4234	4159	4074	4127	4308	4180	4578	4385	4371	4120	4497	4390
	Sulphur (mg kg^{-1})											
0	751	816	796	876	895	827	751	816	796	876	895	827
1 PSI	797	798	843	982	1045	893	808	853	860	791	803	823
2 PSI	772	984	973	1093	913	947	844	892	909	884	862	878
Mean	773	866	871	984	951	889	801	854	855	850	853	842
	Magnesium (mg kg^{-1})											
0	1114	1155	1086	1158	1174	1137	1114	1155	1086	1158	1174	1137
1 PSI	933	913	892	1118	1065	984	871	988	999	959	924	948
2 PSI	1020	1072	1234	1062	1004	1078	870	743	786	746	721	773
Mean	1022	1047	1071	1113	1081	1066	952	962	957	954	940	953

LSD ($P<0.05$)	N	P	K	S	Mg
Type of effluent	NS	123.16	187	33.62	32.20
Levels of effluent	0.04	150.84	229	41.18	46.79
Interaction between type and level of effluent	NS	218.32	324	NS	NS
Overall mean of inorganic fertilizer levels	0.06	104.73	NS	53.16	66.17
Interaction between type of effluent and inorg. fert. levels	0.08	275.40	NS	75.18	NS
Interaction between levels of effluent and inorg. fert. levels	0.102	337.29	NS	NS	104.62
Overall interaction	0.144	NS	NS	NS	NS

PSI: Pre-sowing irrigation; Recommended NPK rate (mg kg^{-1} soil): 48–12–12; NS: Non-significant

In comparison to PREME, a significantly higher content of P, S and Mg was recorded in both rice grain and straw due to the application of POME (Tables 3, 6). The reverse was true of the K content. With an increase in the level of PREME from 0 to 2 PSI, a significant increase was observed in the K (11%) and S (10%) content of rice grain (Table 3), but that of P and Mg decreased by 16 and 19%, respectively. Compared to the control, the application of different levels of inorganic fertilizers significantly increased the contents of P and S in rice plants, whereas the contents of K and Mg remained unaffected.

Table 4

Effect of pre-sowing irrigation with distillery effluent and inorganic fertilizer levels on the micronutrient content (mg kg^{-1}) of rice grain

Effluent levels	Inorganic fertilizer levels (% of recommended NPK)											
	0	25	50	75	100	Mean	0	25	50	75	100	Mean
	Postmethanation effluent (POME)						Premethanation effluent (PREME)					
						Zinc						
0	33.5	42.3	40.0	35.4	40.9	38.4	33.5	42.3	40.0	35.4	40.9	38.4
1 PSI	41.1	46.3	30.2	38.3	38.4	38.9	41.1	32.8	38.9	38.6	35.4	37.3
2 PSI	32.3	39.4	44.6	42.4	42.4	40.2	46.0	39.5	47.7	42.8	40.4	43.3
Mean	35.6	42.7	38.3	38.7	40.6	39.2	40.2	38.2	42.2	38.9	38.9	39.7
						Copper						
0	20.9	16.6	15.1	18.0	18.6	17.8	20.9	16.6	15.1	18.0	18.6	17.8
1 PSI	20.8	22.3	14.6	18.8	20.6	19.4	23.5	15.0	19.3	17.9	19.6	19.1
2 PSI	21.0	14.1	16.2	21.1	20.5	18.6	24.9	25.8	29.6	19.9	22.6	24.6
Mean	20.9	17.7	15.3	19.3	19.9	18.6	23.1	19.4	21.3	18.6	20.3	20.5
						Iron						
0	48.2	42.7	41.3	37.1	47.5	43.4	48.2	42.7	41.3	37.1	47.5	43.4
1 PSI	40.1	48.7	38.9	39.0	44.9	42.3	61.2	52.2	55.4	72.3	64.2	61.1
2 PSI	35.7	39.1	48.7	44.5	35.5	40.7	40.2	32.7	38.4	38.9	66.7	43.4
Mean	41.3	43.5	43.0	40.2	42.6	42.1	49.9	42.6	45.0	49.4	59.5	49.3
						Manganese						
0	44.4	55.9	42.3	43.3	46.0	46.4	44.4	55.9	42.3	43.3	46.0	46.4
1 PSI	35.7	36.2	38.9	54.6	46.1	42.3	48.0	42.2	42.1	48.1	40.7	44.2
2 PSI	45.7	38.4	41.9	48.2	43.1	43.5	47.9	48.0	46.3	35.2	39.7	43.4
Mean	42.0	43.5	41.0	48.7	45.1	44.1	46.8	48.7	43.6	42.2	42.2	44.9
						Molybdenum						
0	2.22	2.14	3.12	2.88	2.14	2.50	2.22	2.14	3.12	2.88	2.14	2.50
1 PSI	2.89	3.21	2.99	2.79	2.46	2.87	3.37	3.29	4.61	3.01	2.79	3.41
2 PSI	2.06	1.73	1.89	2.66	2.61	2.19	3.20	3.93	2.71	2.80	3.53	3.23
Mean	2.39	2.36	2.67	2.78	2.40	2.52	2.93	3.12	3.48	2.90	2.82	3.05
						Boron						
0	5.59	5.70	3.90	3.99	4.04	4.64	5.59	5.70	3.90	3.99	4.04	4.64
1 PSI	4.04	4.04	4.63	4.49	4.63	4.37	4.88	4.93	4.80	4.48	3.41	4.50
2 PSI	4.29	3.42	3.47	4.43	4.05	3.93	4.61	4.18	4.16	4.31	4.46	4.34
Mean	4.64	4.39	4.00	4.30	4.24	4.31	5.03	4.94	4.29	4.26	3.97	4.49

LSD (P<0.05)	Zn	Cu	Fe	Mn	Mo	B
Type of effluent	NS	1.5	3.5	NS	0.24	NS
Levels of effluent	2.5	1.8	NS	NS	0.29	NS
Interaction between type and level of effluent	3.6	2.6	6.0	NS	NS	0.68
Overall mean of inorganic fertilizer levels	NS	2.3	5.5	NS	0.41	NS
Interaction between type of effluent and inorg. fert. levels	4.6	NS	7.8	NS	NS	NS
Interaction between levels of effluent and inorg. fert. levels	5.7	NS	NS	8.7	0.65	1.18
Overall interaction	NS	5.7	13.4	NS	NS	NS

PSI: Pre-sowing irrigation; Recommended NPK rate (mg kg^{-1} soil): 48–12–12; NS: Non-significant

Table 5

Effect of pre-sowing irrigation with distillery effluent and inorganic fertilizer levels on the heavy metal content (mg kg^{-1}) of rice grain

Effluent levels	Inorganic fertilizer levels (% of recommended NPK)											
	0	25	50	75	100	Mean	0	25	50	75	100	Mean
	Postmethanation effluent (POME)						Premethanation effluent (PREME)					
	Lead											
0	2.1	3.5	2.7	2.9	3.7	3.0	2.1	3.5	2.7	2.9	3.7	3.0
1 PSI	3.9	3.9	2.4	4.4	3.3	3.6	4.1	2.2	3.1	2.7	3.4	3.1
2 PSI	4.2	2.3	3.0	3.7	4.5	3.5	3.1	3.6	4.4	3.7	3.8	3.7
Mean	3.4	3.2	2.7	3.7	3.8	3.4	3.1	3.1	3.5	3.1	3.6	3.3
	Cadmium											
0	0.07	0.04	0.05	0.05	0.05	0.05	0.07	0.04	0.05	0.05	0.05	0.05
1 PSI	0.05	0.03	0.05	0.03	0.04	0.04	0.03	0.06	0.04	0.04	0.06	0.05
2 PSI	0.05	0.05	0.03	0.05	0.06	0.05	0.04	0.02	0.04	0.03	0.05	0.04
Mean	0.06	0.04	0.04	0.05	0.05	0.05	0.05	0.04	0.04	0.04	0.05	0.04
	Nickel											
0	0.54	0.51	0.39	0.25	0.29	0.40	0.54	0.51	0.39	0.25	0.29	0.40
1 PSI	0.21	0.29	0.25	0.30	0.26	0.26	0.47	0.41	0.41	0.31	0.48	0.42
2 PSI	0.38	0.36	0.48	0.49	0.41	0.42	0.49	0.47	0.37	0.26	0.27	0.37
Mean	0.38	0.39	0.37	0.35	0.32	0.36	0.50	0.46	0.39	0.27	0.35	0.39
	Chromium											
0	0.91	0.55	0.34	0.33	0.71	0.57	0.91	0.55	0.34	0.33	0.71	0.57
1 PSI	0.54	0.28	0.41	0.50	0.60	0.47	0.67	1.19	0.99	0.99	0.66	0.90
2 PSI	0.84	0.70	0.59	0.77	0.38	0.66	0.32	0.48	0.40	0.47	0.67	0.47
Mean	0.76	0.51	0.45	0.53	0.56	0.56	0.63	0.74	0.58	0.60	0.68	0.65

LSD ($P<0.05$)	Lead	Cadmium	Nickel	Chromium
Type of effluent	NS	NS	NS	0.08
Levels of effluent	NS	NS	0.05	0.09
Interaction between type and level of effluent	NS	NS	0.07	0.14
Overall mean of inorganic fertilizer levels	NS	NS	0.06	0.13
Interaction between type of effluent and inorg. fert. levels	NS	NS	0.09	NS
Interaction between levels of effluent and inorg. fert. levels	NS	NS	0.01	0.22
Overall interaction	NS	NS	NS	0.31

PSI: Pre-sowing irrigation; Recommended NPK rate (mg kg^{-1} soil): 48–12–12; NS: Non-significant

No significant differences in the Zn, Mn and B content of the grain (Table 4) or straw (Table 7) were observed due to the type of effluent applied. Compared to the control, the application of 1 PSI with effluent had no significant effect on the Zn content, but at 2 PSI there was a significant increase in rice grains. In general, the Zn content of the grain did not vary with an increase in the level of fertilizer applied, but it increased significantly in rice straw. As the level of pre-sowing irrigation with effluent increased to 2, a decrease in the Mn and B content of grain was observed (6.7% and 11%, respectively). Different levels of inorganic fertilizers caused no significant differences in the Mn content. As the level of fertilizer application increased, the B content of the grain decreased progressively, but statistically significant differences in B content were recorded only at or above 50% recommended fertilizers.

Table 6

Effect of pre-sowing irrigation with distillery effluent and inorganic fertilizer levels on the macronutrient content of rice straw

Effluent levels	Inorganic fertilizer levels (% of recommended NPK)											
	0	25	50	75	100	Mean	0	25	50	75	100	Mean
	Postmethanation effluent (POME)						Premethanation effluent (PREME)					
	Nitrogen (%)											
0	0.27	0.30	0.38	0.43	0.45	0.37	0.27	0.30	0.38	0.43	0.45	0.37
1 PSI	0.23	0.27	0.36	0.54	0.54	0.39	0.32	0.31	0.23	0.27	0.28	0.28
2 PSI	0.26	0.38	0.40	0.43	0.42	0.38	0.37	0.33	0.41	0.43	0.46	0.40
Mean	0.25	0.32	0.38	0.47	0.47	0.38	0.32	0.31	0.34	0.38	0.40	0.35
	Phosphorus (mg kg^{-1})											
0	1656	1988	1863	2073	2041	1924	1656	1988	1863	2073	2041	1924
1 PSI	1609	1778	2348	2124	2141	2000	2380	2443	1638	1609	1672	1948
2 PSI	2229	2132	2377	2353	2298	2278	1500	1287	1213	1373	1445	1364
Mean	1831	2033	2263	2283	2260	2067	1845	1973	1638	1785	1819	1745
	Potassium (mg kg^{-1})											
0	1.82	1.98	2.38	2.27	2.43	2.18	1.82	1.98	2.38	2.27	2.43	2.18
1 PSI	2.73	3.00	2.35	1.83	1.84	2.35	1.93	2.04	2.83	2.81	2.80	2.48
2 PSI	2.73	2.45	2.53	2.35	2.61	2.53	2.72	3.00	3.21	3.24	3.07	3.05
Mean	2.43	2.48	2.42	2.15	2.29	2.35	2.16	2.34	2.81	2.77	2.77	2.57
	Sulphur (mg kg^{-1})											
0	908	1003	1407	1153	1272	1149	908	1003	1407	1153	1272	1149
1 PSI	1261	1238	1880	1353	1826	1512	1683	1560	1145	983	1169	1308
2 PSI	1395	1620	1871	1922	1679	1697	1459	1216	1305	1170	1514	1333
Mean	1188	1287	1719	1476	1592	1453	1350	1260	1286	1102	1318	1263
	Magnesium (mg kg^{-1})											
0	1.25	1.28	1.31	1.35	1.32	1.30	1.25	1.28	1.31	1.35	1.32	1.30
1 PSI	1.31	1.34	1.31	1.33	2.08	1.47	1.93	2.04	2.83	2.72	2.80	2.46
2 PSI	2.73	3.00	2.35	1.80	1.84	2.34	3.56	3.21	3.37	3.24	3.07	3.29
Mean	1.76	1.87	1.66	1.49	1.75	1.70	2.25	2.17	2.50	2.44	2.40	2.35

LSD ($P<0.05$)	N	P	K	S	Mg
Type of effluent	NS	65.16	0.149	97.30	0.132
Levels of effluent	NS	83.48	0.183	119.17	0.161
Interaction between type and level of effluent	0.07	118.05	0.258	168.54	NS
Overall mean of inorganic fertilizer levels	0.06	107.77	NS	153.85	0.223
Interaction between type of effluent and inorg. fert. levels	NS	152.41	0.333	217.58	0.295
Interaction between levels of effluent and inorg. fert. levels	NS	186.66	NS	NS	NS
Overall interaction	NS	263.98	0.577	376.86	0.511

PSI: Pre-sowing irrigation; Recommended NPK rate (mg kg^{-1} soil): 48–12–12; NS: Non-significant

In both rice grain and straw, the Cu content was found to be higher after the application of POME as compared to PREME (Tables 4, 7). As the level of inorganic fertilizers increased, the Cu content of rice grain decreased significantly, while that of straw remained unaffected. A significantly higher Fe content was observed in the grains after PREME application compared to POME. In the case of PREME, a significant increase in Fe content was recorded at 1 PSI, whereas it decreased significantly at 2 PSI compared to the control. A significant difference in the Mo content due to the type of effluent was only observed in the grain. In general, the Mo content of rice grain (2.5 mg kg^{-1}) grown with POME was found to be significantly lower than for that grown with PREME (3.1 mg kg^{-1}). However, the increase in the Mo contents in rice grain and straw as the effluent level rose was more significant for PREME than for POME.

Table 7

Effect of pre-sowing irrigation with distillery effluent and inorganic fertilizer levels on the micronutrient content (mg kg^{-1}) of rice straw

Effluent levels	Inorganic fertilizer levels (% of recommended NPK)											
	0	25	50	75	100	Mean	0	25	50	75	100	Mean
	Postmethanation effluent (POME)						Premethanation effluent (PREME)					
Zinc												
0	24.3	27.8	34.5	29.8	32.2	29.7	24.3	27.8	34.5	29.8	32.2	29.7
1 PSI	24.5	23.9	33.7	31.5	44.3	31.6	38.5	37.6	25.9	25.1	27.8	31.0
2 PSI	28.1	43.8	43.6	31.3	29.2	35.2	23.2	27.4	22.8	26.7	32.0	26.4
Mean	25.6	31.8	37.3	30.9	35.3	32.2	28.6	30.9	27.8	27.2	30.7	29.0
Copper												
0	4.1	5.8	3.3	4.2	2.6	4.0	4.1	5.8	3.3	4.2	2.6	4.0
1 PSI	6.4	5.6	7.8	7.2	6.5	6.7	6.2	7.4	5.9	5.4	8.7	6.7
2 PSI	7.3	7.6	8.5	6.6	6.8	7.4	6.6	5.9	4.4	5.3	6.6	5.8
Mean	5.9	6.3	6.5	6.0	5.3	6.0	5.6	6.3	4.6	5.0	6.0	5.5
Iron												
0	4.1	5.8	3.3	4.2	2.6	4.0	4.1	5.8	3.3	4.2	2.6	4.0
1 PSI	6.4	5.6	7.8	7.2	6.5	6.7	6.2	7.4	5.9	5.4	8.7	6.7
2 PSI	7.3	7.6	8.5	6.6	6.8	7.4	6.6	5.9	4.4	5.3	6.6	5.8
Mean	5.9	6.3	6.5	6.0	5.3	6.0	5.6	6.3	4.6	5.0	6.0	5.5
Manganese												
0	175	138	174	131	178	159	175	138	174	131	178	159
1 PSI	147	134	212	141	156	158	152	148	120	145	160	145
2 PSI	178	222	151	131	160	168	182	130	127	148	169	151
Mean	167	165	179	134	165	162	170	139	140	141	169	152
Molybdenum												
0	3.2	2.8	3.1	3.3	3.3	3.1	3.2	2.8	3.1	3.3	3.3	3.1
1 PSI	1.6	4.8	1.2	4.4	3.7	3.1	3.7	5.6	3.8	4.6	3.1	4.1
2 PSI	3.9	3.8	3.1	6.4	4.6	4.3	3.8	4.4	5.1	4.6	5.3	4.6
Mean	2.9	3.8	2.5	4.7	3.8	3.5	3.6	4.3	4.0	4.2	3.9	4.0
Boron												
0	11.7	12.8	15.9	13.4	11.8	13.1	11.7	12.8	15.9	13.4	11.8	13.1
1 PSI	14.0	12.3	17.9	12.7	11.6	13.7	11.9	16.8	11.9	12.2	16.2	13.8
2 PSI	10.6	15.6	14.1	17.9	12.5	14.1	13.1	15.6	12.9	13.2	16.5	14.3
Mean	12.1	13.6	15.9	14.7	11.9	13.7	12.2	15.1	13.6	12.9	14.8	13.7

LSD ($P<0.05$)	Zn	Cu	Fe	Mn	Mo	B
Type of effluent	2.4	0.5	NS	21.84	NS	NS
Levels of effluent	NS	0.6	NS	NS	0.5	NS
Interaction between type and level of effluent	4.2	0.8	NS	37.84	0.7	NS
Overall mean of inorganic fertilizer levels	3.8	NS	19.9	NS	NS	1.35
Interaction between type of effluent and inorg. fert. levels	5.4	1.0	NS	48.85	NS	1.91
Interaction between levels of effluent and inorg. fert. levels	NS	1.3	NS	NS	1.2	3.30
Overall interaction	9.4	NS	48.6	84.6	NS	2.33

PSI: Pre-sowing irrigation; Recommended NPK rate (mg kg^{-1} soil): 48–12–12; NS: Non-significant

In comparison to the grain, a relatively greater content of heavy metals was observed in the straw. Under different treatment combinations, the contents of Pb, Ni, Cr and Cd ranged from 2.1–4.5, 0.2–0.5, 0.3–1.2 and 0.02-0.07 mg kg^{-1} in the grain and from 1.7–5.6, 1.0–2.3, 1.5–3.1 and 0.06–0.18 mg kg^{-1}, respectively, in the straw (Tables 5, 8). The Cr content of the grain increased significantly with the application of PREME and that of Pb in the straw with the application of POME. The heavy metal content of rice straw decreased significantly as the level of effluent increased from 0 to 1 PSI, but a further increase in the level of effluent to 2 PSI led to a significant increase in their content. As the level of inorganic fertilizer increased to 50% of the recommended level, a significant depression in the Ni and Cr contents of rice grain was observed. In the case of PREME, an increase in the level of effluent resulted in a non-significant increase in Pb content and a non-significant decrease in Cd. A significant decrease in the Ni and Cr contents of the grain was observed at 2 PSI, and a significant decrease in Pb and Ni at 1 PSI in the straw. The interaction between the type of effluent and the inorganic fertilizer level was non-significant for the Pb, Cd, Ni and Cr contents of the straw, but was significant for the Ni content of the grain. The interaction between the fertilizer and effluent levels was found to be significant for the Pb, Ni and Cr contents of the straw and the Ni and Cr contents of the grain. In the present investigation, the heavy metal contents in rice grain and straw were well below the critical concentrations suggested by Alloway (1990) for plants. Although there is some evidence suggesting a beneficial role for some heavy metals, such as Ni, Cr and Cd, in animal and human nutrition, enough data does not exist to define their specific roles with certainty. In fact, plants do not necessarily act as an indicator of levels for animals and humans, since plants can tolerate higher levels of heavy metals than animals and humans. Information regarding diet standards with respect to heavy metals for animal/human nutrition is scanty. The yield of rice is not reduced by Cd toxicity until the concentration of Cd is many times higher than the 1 mg Cd kg^{-1} maximum permissible concentration for human consumption (Asami, 1984). Safe, adequate intake levels for Ni may range between 0.1 and 0.3 mg day^{-1} for humans, but conventional diets often provide less than 0.15 mg day^{-1} (Nielsen et al., 1990). Livestock grazing on herbage containing 10–50 mg Mo kg^{-1} may suffer from molybdenum toxicity (Kubota, 1975). Extensive risk evaluation studies suggest that the greatest risk for human exposure to lead comes from direct particulate ingestion and not food chain transfer (Chaney and Ryan, 1994).

In general, the interaction between types and levels of effluents was significant for most of the nutrients, including P, K, Zn, Cu, Fe, B, Ni and Cr in grain and N, P, K, S, Zn, Cu, Mn, Pb and Ni in straw. The interaction between type of effluents and inorganic fertilizers was significant for N, P, S, Zn, Fe and Ni in the grain and for P, K, S, Mg, Zn, Cu, Mn and B in the straw, while the interaction effect of levels of effluents versus inorganic fertilizer was significant for N, P, Mg, Zn, Mn, Mo, B, Ni and Cr in the grain and for P, Cu, Mo, B, Pb, Ni and Cr in the straw. The significant interactions between these parameters indicated that nutrient availability and absorption is affected differently by different types and levels of effluents and rates of inorganic fertilizers.

Compared with the critical levels reported by Yoshida (1981) for P, K, Mg, Cu, Zn and B and by Osiname and Kang (1975) for S, all the elements were in the satisfactory range.

Table 8

Effect of pre-sowing irrigation with distillery effluent and inorganic fertilizer levels on the heavy metal content (mg kg^{-1}) of rice straw

Effluent levels	Inorganic fertilizer levels (% of recommended NPK)											
	Postmethanation effluent (POME)						Premethanation effluent (PREME)					
	0	25	50	75	100	Mean	0	25	50	75	100	Mean
						Lead						
0	3.77	4.32	3.81	4.11	4.74	4.15	3.77	4.32	3.81	4.11	4.74	4.15
1 PSI	3.71	2.11	3.67	2.33	2.08	2.78	2.11	2.52	3.26	2.67	1.88	2.49
2 PSI	4.61	4.06	4.57	4.52	5.63	4.68	5.09	1.96	1.68	2.23	3.08	2.81
Mean	4.03	3.50	4.02	3.65	4.15	3.87	3.66	2.93	2.92	3.00	3.23	3.15
						Cadmium						
0	0.06	0.08	0.08	0.08	0.12	0.08	0.06	0.08	0.08	0.08	0.12	0.08
1 PSI	0.13	0.11	0.15	0.10	0.10	0.12	0.12	0.13	0.11	0.12	0.14	0.12
2 PSI	0.13	0.18	0.15	0.12	0.15	0.15	0.13	0.11	0.09	0.12	0.13	0.12
Mean	0.11	0.12	0.13	0.10	0.12	0.12	0.10	0.11	0.09	0.11	0.13	0.11
						Nickel						
0	1.16	1.47	2.35	1.68	2.08	1.75	1.16	1.47	2.35	1.68	2.08	1.75
1 PSI	1.40	1.29	1.54	0.82	1.21	1.25	1.45	1.38	1.64	1.25	1.04	1.35
2 PSI	1.90	2.02	2.15	2.10	2.35	2.10	1.53	1.37	1.50	2.19	1.76	1.67
Mean	1.49	1.59	2.01	1.53	1.88	1.70	1.38	1.41	1.83	1.71	1.63	1.59
						Chromium						
0	2.11	2.21	3.07	2.66	2.97	2.60	2.11	2.21	3.07	2.66	2.97	2.60
1 PSI	2.51	2.29	2.33	1.57	1.48	2.04	1.74	1.57	2.36	1.53	1.98	1.84
2 PSI	2.61	2.67	2.80	3.07	3.25	2.88	2.42	2.13	2.33	3.43	2.39	2.54
Mean	2.41	2.39	2.73	2.43	2.57	2.51	2.09	1.97	2.59	2.54	2.45	2.33

LSD ($P<0.05$)	Lead	Cadmium	Nickel	Chromium
Type of effluent	0.25	NS	NS	NS
Levels of effluent	0.31	0.017	0.17	0.24
Interaction between type and level of effluent	0.44	NS	0.25	NS
Overall mean of inorganic fertilizer levels	0.40	NS	0.22	0.31
Interaction between type of effluent and inorg fert levels	NS	NS	NS	NS
Interaction between levels of effluent and inorg fert levels	0.69	NS	0.39	0.53
Overall interaction	0.98	NS	NS	NS

PSI: Pre-sowing irrigation; Recommended NPK rate (mg kg^{-1} soil): 48–12–12; NS: Non-significant

Conclusions

One pre-sowing irrigation with postmethanation distillery effluent along with inorganic fertilizers proved most effective in increasing the grain and straw yield of rice. Compared to premethanation distillery effluent, the application of inorganic fertilizer in the presence of postmethanation effluent was highly beneficial to the rice crop. The application of distillery effluent led to significant changes in the composition of major and minor nutrients in both rice grain and straw. The heavy metal contents in the rice grain and straw were well below the critical limits for plants.

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STUDY ON DROUGHT RESISTANCE INDICES IN SPRING SAFFLOWER

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Safflower (*Carthamus tinctorius* L.) is a native Iranian oilseed crop adapted to various environments in the country. It can be planted as a winter or spring crop. The calculation of drought resistance indices could help us to identify drought-resistant genotypes. To determine these yield-based indices, an investigation was carried out with 16 safflower varieties/lines in spring planting at six research stations located at Sararood, Maragheh, Ghamlo, Zanjan, Shirvan and Kohdasht in 2001. The experiments were laid out in a random complete block design under drought-stressed and non-stressed conditions with three replications. Drought resistance indices, including MP, GMP, TOL, STI and SSI, were calculated from the seed yield data. The genotypes were planted in a greenhouse and the cell membrane stability (CMS) was measured using PEG solutions. Analysis of variance revealed significant differences for all indices between the locations, so experiments should be conducted at different locations over several years for the accurate estimation of drought resistance indices. STI was the best index to identify superior genotypes in conditions both with and without drought stress. The estimation of STI from the mean of all locations showed that Gila had the highest STI (0.430), with high seed yield under both conditions. Analysis of variance showed significant differences between the genotypes for CMS at the 1% level of probability, with the highest value for S-541 and the lowest for Kino-76. There were significant and strong correlations between STI, MP and GMP with CMS, so cell membrane stability could be useful as a fast, cheap method for screening germplasm and identifying drought-resistant genotypes. Cluster analysis on the basis of STI, MP, GMP, CMS and seed yield under both stressed and non-stressed conditions divided the genotypes into three groups.

Key words: safflower, water stress, cell membrane stability, seed yield

Introduction

Safflower (*Carthamus tinctorius* L.) is a multi-purpose crop and well adapted to Iranian conditions. Ashri (1973) reported that Iran, Afghanistan, the Near East and Turkey were the centre of diversity for safflower. In Egypt dye from safflower was used to colour cotton and silk (Weiss, 1971) and in China a herbal tea is prepared from safflower blossoms (Dajue and Yuanzhou; 1993). The deep root system of safflower enables the plant to draw moisture and nutrients from a considerable depth of soil (Dajue and Mundel, 1996, Mundel et al., 1995). It is a suitable crop for rotation with wheat (Pourdad and Beg, 2003) and barley (Yau, 2005) under Mediterranean rain-fed conditions. The development of drought-resistant cultivars, however, is hampered by the low heritability of drought tolerance and a lack of effective selection strategies (Kirigwi et al., 2004). Selection for high seed yield under non-stressed conditions was not effective to find drought-resistant genotypes (Blum, 1979; Ceccarelli and Grando, 1991; Rathjen, 1994). On the other hand, some researchers proposed that selection should be done under both stress and non-stress conditions (Sinmena et al., 1993; Rajaram and Van Ginkle, 2001; Betran et al., 2003). Genotypes having integrated desirable alleles with superior performance can be obtained by selection under both types of conditions (Richards, 1996). The difference between seed yield under stressed and non-stressed conditions is called tolerance (TOL) and also known as the drought resistance index (Rosielle and Hamblin, 1981). Genotypes selected on the basis of this index have relatively high seed yield under stressed conditions and low productivity under non-stressed conditions. The mean yield over the two types of conditions is known as the Mean of Productivity (MP) (Rosielle and Hamblin, 1981). Fernandez (1992) proposed the Stress Tolerance Index (STI) as a criterion to select drought-resistant genotypes. Genotypes with high STI are superior in performance under both stressed and non-stressed conditions. This author suggested the Geometric Mean of Productivity (GMP) as another useful criterion. The Stress Susceptibility Index (SSI) was developed by Fischer and Maurer (1978) as a drought selection index; low SSI indicates that there is little difference between the productivity under stressed and non-stressed conditions.

Cell membrane is one of the first plant tissues to be injured by drought stress (Levitt, 1980). The transfer of electrolytes is disturbed as a consequence of cell membrane leakage (Blum and Ebercon, 1980). There are several different procedures for measuring cell membrane stability (CMS), which can be related to drought resistance (Bandurska, 2000; Venkateswarlu and Ramesh, 1993). Kocheva and Georgiev (2003) showed that drought-resistant genotypes of barley suffered less cell membrane damage as compared to susceptible genotypes.

These indices have been measured by many researchers in different crops, but at single locations, making the results of restricted application.

The objective of this study was to compare different drought resistance indices over several locations to increase the reliability of the results and to select the best index.

Materials and methods

In this study 16 safflower (*Carthamus tinctorius* L.) lines and varieties (Table 1a, b) were planted under moisture-stressed and non-stressed conditions at six research stations in Iran, including Sararood in the mid-west in the foothills of the Zargos mountains, which is a semi-cold region; Maragheh in the north-west cold region; Ghamlo in the mid-west cold region; Zanjan in the north-west cold region; Shirvan in the north-east, mostly semi-cold region; and Kohdasht in the extreme mid-west, which has semi-cold and warm areas. The trials were conducted in 2001 in a randomized complete block design (RCBD) with three replications. Sowing was done by hand in plots with five rows 6 m in length and 30 cm apart. The seeding rate was 30 seeds m^{-2} for all locations. Fertilizer (40 kg ha^{-1} nitrogen and 60 kg ha^{-1} P_2O_5) was applied before planting. The yield (kg ha^{-1}) was obtained by converting the seed yield per plot to hectares. Non-stressed plots were irrigated three times, at the bud formation, flowering and grain filling stages, while stressed plots received no water other than rainfall. Precipitation data and location descriptions are given in Table 1a, b. Five drought resistance indices were calculated as below:

1. Stress Susceptibility Index

$$SSI = \frac{1-(Ys/Yp)}{1-(\overline{Ys}/\overline{Yp})}$$ (Fischer and Maurer, 1978)

2. Tolerance

$$TOL = Yp - Ys$$ (Rosielle and Hamblin, 1981)

3. Mean of Productivity

$$MP = \frac{(Yp+Ys)}{2}$$ (Rosielle and Hamblin, 1981)

4. Geometric Mean of Productivity

$$GMP = \sqrt{(Y_s)(Y_p)}$$ (Fernandez, 1992)

5. Stress Tolerance Index

$$STI = \left(\frac{Y_p}{\overline{Y}_p}\right)\left(\frac{Y_s}{\overline{Y}_s}\right)\left(\frac{\overline{Y}_s}{\overline{Y}_p}\right) = \frac{(Y_p)(Y_s)}{(\overline{Y}_p^2)}$$ (Fernandez, 1992)

where:

Y_s: yield of a given genotype under stressed conditions; Y_p: yield of a given genotype under non-stressed conditions. $\overline{Y}_s$ mean yield under stressed conditions; $\overline{Y}_p$: mean yield under non-stressed conditions.

To measure cell membrane stability, an experiment was conducted with 16 genotypes in a randomized complete design (RCD) with three replications in a greenhouse. Twenty seeds from each were planted in pots at Sararood in April 2001. Leaf slices of the same size were cut from each genotype and put in 0% or 40% PEG (polyethylene glycol) solution at 25°C for 24 hours. Then electroconductivity (EC) was measured (first reading). The leaf samples were then autoclaved for 20 min at 120°C prior to the second reading.

Cell membrane stability (CMS) was calculated as follows:

$$CMS = \frac{1-\frac{T_1}{T_2}}{1-\frac{C_1}{C_2}}$$ (Blum and Ebercon, 1980)

where :

T_1: First EC reading in 40% PEG solution; T_2: second EC reading in 40% PEG solution; C_1: first EC reading in 0% PEG solution; C_2: second EC reading in 0% PEG solution.

Analysis of variance was made for the drought resistance indices, taking the locations as replications. The genotypes were grouped on the basis of the five drought resistance indices and CMS using the UPGMA method of cluster analysis.

Table 1a
Origin and characters of genotypes

No.	Genotypes	Origin	Appearance
1	LRV-51-51	Iran	Spiny, yellow florets
2	Cyprobregon	Cyprus	Spiny, yellow florets
3	Hartman	USA	Spiny, yellow florets
4	697	Iran	Spineless, red florets
5	Kino-76	ICARDA	Spiny, yellow florets
6	S-541	USA	Spiny, red florets
7	Syrian	Syria	Spineless, red florets
8	Dincer	Turkey	Spiny, yellow-orange florets
9	PI-250537	World Bank of Safflower	Spiny, yellow-orange florets
10	PI-537598	USA	Spiny, yellow-orange florets
11	Isfahan Local	Iran	Spineless, red florets
12	PI-250536	World Bank of Safflower	Spiny, yellow-orange florets
13	CW-4440	USA	Spiny, yellow-orange florets
14	Lesaf	–	Spiny, yellow florets
15	CW-74	USA	Spiny, yellow florets
16	Gila	USA	Spiny, yellow-red florets

Table 1b
Annual precipitation and site conditions

Locations	Rainfall (mm)	Altitude (m)	Soil type
Sararood	440.6	1351	Silty clay
Maragheh	381.8	1400	Clay-loam
Ghamlo	350.3	1850	Clay-loam
Zanjan	383.3	1875	Clay-loam
Shirvan	329.6	1131	Clay-loam
Kohdasht	386.3	1200	Loam

Results and discussion

Drought resistance indices were calculated for all the genotypes at each location, taking the locations as replications. Analysis of variance (Table 2) showed that there were significant differences between the locations for all the drought resistance indices except SSI. This confirmed that the estimation of these indices at a single location cannot be generalized to give estimates for other locations. To obtain more reliable results, SSI should be estimated over several locations. Differences between the genotypes were significant for MP and STI, but non-significant for the other three indices. The mean of the yield-based indices (Table 3) showed that the variety Gila had the highest STI, MP and GMP, giving a high yield under both stressed (618.1 kg/ha) and non-stressed (1855.7 kg/ha) conditions. On the other hand, Kino-76, which had the lowest STI and GMP, gave the lowest yield when stressed and a low yield under non-stressed conditions. The results clearly revealed that STI was able to identify genotypes with high yield under both stressed and non-stressed conditions and to differentiate drought-resistant from drought-susceptible genotypes. Liravi (2005) suggested that STI and TOL were suitable indices for selecting drought-resistant genotypes in winter safflower. A study on the inheritance of yield-based drought resistance indices in winter wheat showed that STI and the standard superiority measure (SP) (Fox and Rosielle, 1982) had higher narrow-sense heritability than other indices and were better indices to employ in plant breeding programmes (Saba et al., 2001).

The mean yield under both conditions and the STI of genotypes at six locations (Tables 4a,b) showed that the yield under stressed conditions was always lower than under non-stressed conditions. Substantial differences were found, as also reported by other researchers in safflower (El-Wakil et al., 1989; Patil et al., 1992). STI was high at most of the locations for Gila, CW-4440 and PI-537598, but the standard deviation of STI was lower in CW-4440 than for the other two genotypes, which indicated greater stability for STI in this genotype.

Table 2

Analysis of variance (mean of squares) for yield-based drought resistance indices in 16 safflower varieties/lines

S.O.V.	DF	Mean of squares				
		STI	TOL	SSI	GMP	MP
Replication	5	1.27**	14244438.1**	0.008 ns	1111917.2**	2673878.3**
Genotype	15	0.025**	71298.9 ns	0.171 ns	18485.1 ns	34787.0*
Error	75	0.015	59827.8	0.140	11647/5	17188.6

STI: Stress Tolerance Index; TOL: Tolerance; SSI: Stress Susceptibility Index; GMP: Geometric Mean of Productivity; MP: Mean of Productivity; * and** are significant at 5 and1%, respectively; NS: Non-significant

Table 3

Means of yield-based drought resistance indices in 16 safflower varieties/lines

Genotype	TOL	SSI	STI	GMP	MP	Ys	Yp
LRV-51-51	1066.35	1.001	0.353	971.33	1108.03	574.87	1641.20
Cyprobregon	1126.93	1.046	0.331	940.02	1095.95	532.50	1659.40
Hartman	1034.33	1.020	0.309	908.16	1045.08	527.92	1562.25
697	1039.47	0.996	0.343	956.43	1088.51	568.78	1608.25
Kino-76	1033.47	1.035	0.290	880.48	1020.91	504.18	1537.65
S-541	1105.17	0.988	0.399	1032.76	1171.30	618.72	1723.88
Syrian	953.25	0.951	0.342	955.72	1067.97	591.35	1544.60
Dincer	841.43	0.923	0.296	888.86	983.40	562.68	1404.12
PI-250537	1101.60	1.008	0.368	990.71	1133.53	582.73	1684.33
PI-537598	1262.48	1.046	0.415	1052.94	1227.66	596.42	1858.90
Isfahan Local	1063.57	0.994	0.360	980.79	1115.68	583.90	1647.47
PI-250536	943.93	0.987	0.292	882.48	1000.76	528.80	1472.73
CW-4440	1100.67	0.967	0.429	1070.64	1203.80	653.47	1754.13
Lesaf	1131.02	1.049	0.329	937.82	1095.12	529.62	1660.63
CW-74	932.30	0.937	0.344	958.48	1065.82	599.67	1531.97
Gila	1237.60	1.027	0.430	1071.04	1236.95	618.15	1855.75

TOL: Tolerance; SSI: Stress Susceptibility Index; STI: Stress Tolerance Index; GMP: Geometric Mean of Productivity; MP: Mean of Productivity; Ys: yield under stressed conditions, Yp: yield under non-stressed conditions

Table 4a

Mean seed yield of safflower genotypes under stressed and non-stressed conditions and STI values at six locations

Genotype	Maragheh			Ghamlo			Kohdasht			Mean^{+}		STD of STI^{+}
	Yp	Ys	STI	Yp	Ys	STI	Yp	Ys	STI	Yp	Ys	
LRV-51-51	1342.6	533.3	0.385	2340.0	513.9	0.274	174307	637.0	0.443	1641.20	574.87	0.240
Cyprobregon	1375.0	522.2	0.386	2397.0	377.7	0.206	1167.4	500.0	0.233	1659.40	532.50	0.256
Hartman	1314.8	440.7	0.312	2123.0	431.6	0.209	1952.6	703.6	0.547	1562.25	527.92	0.208
697	1416.7	527.8	0.402	1875.0	362.1	0.155	1779.0	636.7	0.451	1608.25	568.78	0.277
Kino-76	1287.0	525.9	0.364	1786.0	175.1	0.071	1454.8	651.8	0.378	1537.65	504.18	0.257
S-541	1490.7	561.1	0.450	2417.0	428.5	0.236	1864.4	622.9	0.492	1723.88	618.72	0.385
Syrian	1287.0	516.7	0.358	2404.0	360.6	0.198	1126.6	918.5	0.412	1544.60	591.35	0.246
Dincer	1263.9	594.5	0.404	1318.0	308.9	0.092	1408.1	755.5	0.424	1404.12	562.68	0.308
PI-250537	1240.7	472.2	0.315	2246.0	405.9	0.208	1523.0	562.9	0.342	1684.33	582.73	0.284
PI-537598	1439.8	537.1	0.416	2460.0	449.9	0.252	1834.0	874.0	0.639	1858.90	596.42	0.343
Isfahan local	1467.6	420.4	0.332	2049.0	452.1	0.211	1732.0	874.0	0.603	1647.47	583.90	0.309
PI-250536	1245.4	618.5	0.414	1949.0	282.9	0.126	1103.2	574.0	0.252	1472.73	528.80	0.257
CW-4440	1254.6	492.6	0.333	2636.0	503.4	0.303	1648.0	892.6	0.586	1754.13	653.47	0.285
Lesaf	1495.4	450.0	0.362	2137.0	401.7	0.196	161603	625.9	0.403	1660.63	529.62	0.279
CW-74	1347.2	425.9	0.309	1597.0	535.0	0.195	1699.2	848.0	0.574	1531.97	599.67	0.329
Gila	1541.7	553.7	0.459	1772.0	397.8	0.161	1691.8	848.1	0.572	1855.75	618.15	0.458

$^{+}$: together with Table 4b; Ys: yield under stressed conditions, Yp: yield under non-stressed conditions, STI: Stress tolerance index, STD: Standard deviation

Table 4b

Mean seed yield of safflower genotypes under stressed and non-stressed conditions and STI values at six locations

Genotype	Sararood			Zangan			Shirvan			Mean^{+}		STD of STI^{+}
	Yp	Ys	STI	Yp	Ys	STI	Yp	Ys	STI	Yp	Ys	
LRV-51-51	1080.9	1047.6	0.724	2759.7	378.3	0.117	580.4	339.1	0.71	1641.20	574.87	0.240
Cyprobregon	1300.6	1030.4	0.856	3259.7	482.0	0.176	456.9	282.7	0.47	1659.40	532.50	0.256
Hartman	959.2	890.7	0.546	2519.0	370.7	0.105	504.9	330.2	0.61	1562.25	527.92	0.208
697	1200.0	1177.0	0.903	2963.7	415.3	0.138	415.1	293.8	0.44	1608.25	568.78	0.277
Kino-76	1313.0	962.2	0.807	2982.0	433.7	0.149	403.1	276.4	0.40	1537.65	504.18	0.257
S-541	1238.9	1192.9	0.944	2667.0	415.3	0.124	665.3	451.6	1.09	1723.88	618.72	0.385
Syrian	1176.5	964.4	0.725	2759.7	419.0	0.129	513.8	368.9	0.69	1544.60	591.35	0.246
Dincer	1253.7	1002.6	0.803	2630.3	333.7	0.098	550.7	380.9	0.76	1404.12	562.68	0.308
PI-250537	1133.9	1192.6	0.864	3463.3	463.7	0.180	499.1	399.1	0.72	1684.33	582.73	0.284
PI-537598	1448.7	1024.4	0.948	3348.7	296.7	0.111	622.2	396.4	0.90	1858.90	596.42	0.343
Isfahan local	1303.7	1052.9	0.877	2741.3	356.0	0.109	590.7	348.0	0.75	1647.47	583.90	0.309
PI-250536	1216.1	1052.5	0.797	2926.3	352.3	0.115	396.4	319.6	0.46	1472.73	528.80	0.257
CW-4440	1166.1	1292.2	0.693	3389.0	404.0	0.153	431.1	336.0	0.53	1754.13	653.47	0.285
Lesaf	1355.6	990.0	0.858	2833.7	367.0	0.116	525.8	343.1	0.65	1660.63	529.62	0.279
CW-74	1233.3	1117.4	0.881	2722.7	297.0	0.091	592.4	374.7	0.81	1531.97	599.67	0.329
Gila	1633.3	1307.4	1.36	3845.0	274.3	0.118	650.7	327.6	0.77	1855.75	618.15	0.458

$^{+}$: together with Table 4a; Ys: yield under stressed conditions, Yp: yield under non-stressed conditions, STI: Stress tolerance index, STD: Standard deviation

Based on the yield under stressed and non-stressed conditions and the STI, the genotypes can be divided into four groups: group A included Gila, PI-537598, CW-4440, Isfahan Local, PI-250537, 697, LRV-51-51 and S-541, which produced high yields under both conditions; group B included Syrian, CW-74 and Dincer, which had high yields under stressed conditions; group C included the varieties Lesaf and Cyprobregon, which had high yields under non-stressed conditions; group D included Hartman, Kino-76 and PI-250536, which had low yields under both conditions (Fig. 1).

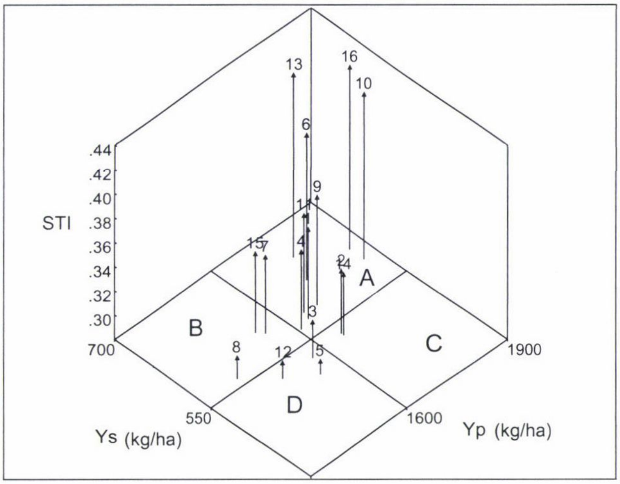

Fig. 1. Mean of yield under stressed and non-stressed conditions and STI in safflower varieties and lines. (For genotypes see Table 1a)

Analysis of variance for cell membrane stability (CMS) showed significant differences between the genotypes (Table 5). The highest and lowest values of CMS were recorded for S-541 and Kino-76, respectively. Gila, PI-537598, CW-4440 and Isfahan Local had higher CMS than the other genotypes (Table 6).

Cluster analysis based on the yield under stressed and non-stressed conditions and on the indices STI, MP, GMP and CMS divided the genotypes into three groups (Table 7). The first cluster included four genotypes with high yields under both conditions, having high drought resistance indices and CMS. The second and third clusters each included six genotypes with low and medium parameters, respectively.

In conclusion, this study showed that to estimate more reliable drought resistance indices trials should be conducted over locations or years. STI was the best index to select drought-resistant safflower genotypes which had high yields under both stressed and non-stressed conditions. Cell membrane stability can be used as a quick and cheap method to screen large numbers of genotypes for drought resistance in safflower breeding programmes. Cluster analysis based on suitable drought resistance indices and CMS can be a useful method for grouping plant materials into different drought resistance clusters. Gila, PI-537598 and CW-4440 were drought-resistant genotypes with high yield under both stressed and non-stressed conditions, but Gila had high standard deviation for STI over locations, so the other two genotypes were more suitable for selection as drought-resistant genotypes.

Table 5

Analysis of variance (mean of squares) for cell membrane stability in 16 safflower varieties/lines

S.O.V.	df	CMS
Replication	2	38.75 ns
Genotype	15	40158.02**
Error	30	22.72

** significant at 1%, respectively; ns: non-significant

Table 6

Mean comparison of cell membrane stability in 16 safflower varieties/lines

No.	Variety/Line	Means
1	LRV-51-51	0.88726 ab
2	Cyprobregon	0.70746 bc
3	Hartman	0.72398 abc
4	697	0.78265 abc
5	Kino-76	0.56566 c
6	S-541	0.97342 a
7	Syrian	0.72941 abc
8	Dincer	0.86455 ab
9	PI-250537	0.8643 ab
10	PI-537598	0.89442 ab
11	Isfahan local	0.89985 ab
12	PI-250536	0.71574 bc
13	CW-4440	0.95813 ab
14	Lesaf	0.71576 bc
15	CW-74	0.86574 ab
16	Gila	0.95343 ab

Different letters represent significant differences at the 5% probability level.

Table 7

Cluster means of cluster analysis on the basis of the indices MP, GMP, STI, CMS and yield under stressed and non-stressed conditions

Genotype	Cluster	Mean of clusters					
		CMS	STI	GMP	MP	Ys	Yp
Gila, PI-537598, CW-4440, S-541	I	0.94485	0.418	1056.8	1209.9	621.7	1798.2
PI-250536, Dincer, CW-74, Syrian, Kion-76, Hartman	II	0.74418	0.312	912.4	1030.7	552.3	1508.9
Lesaf, Cyprobregon, 697, PI -250537, Isfahan Local, LRV-51-51	III	0.80955	0.347	962.85	1106.14	562.07	1650.2

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GENETIC VARIABILITY AND CORRELATION STUDIES IN 'EGUSI' MELON [*Citrullus lanatus* (Thunb.) Matsum. & Nakai]

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Genetic variability and correlation analysis were studied in 20 accessions of 'egusi' melon during two growing seasons. The genotypic correlation coefficients with seed yield were partitioned into direct and indirect effect causes. Heritability in the broad sense ranged from 17% for fruit circumference to 90% for days to germination and flowering in the early season, while in the late season, heritability ranged from 7% for seed weight per fruit to 88% for days to germination. High phenotypic and genotypic coefficients of variation were recorded for seed yield while days to maturity had the lowest in both seasons. Fruit circumference and fruit weight had significant genotypic and phenotypic correlation with seed yield in the early season, while number of branches per plant, vine length per plant, number of fruits per plant and fruit circumference per plant showed significant genotypic and phenotypic correlation with seed yield in the late season. Environmental correlation coefficients were significant between seed yield and vine length per plant, number of fruits per plant and fruit size per plant. Vine length per plant and fruit circumference per plant had the largest positive direct effect on seed yield. Knowledge of the relationship of these characters with seed yield will aid in the selection of genotypes that have high seed yield, which will also be specific to the two major seasons in the year.

Key words: genetic variability, 'egusi' melon, genotypic correlation, phenotypic correlation, environmental correlation, direct effect, coefficient of variation, heritability

Introduction

'Egusi' melon, *Citrullus lanatus* (Thunb.) Matsum. & Nakai, is one of the most important vegetable crops in the tropical and subtropical regions of the world. The mesocarp of the fruits is extremely bitter, but the seeds are important sources of vitamin E and are rich in proteins and oils which can be extracted for cooking purposes. The seeds can also be ground into a powder and used as a soup thickener or flavouring agent (Badifu and Ogunsua, 1991). Melon seed contains about 314 g kg^{-1} DM crude protein, 439.3 g kg^{-1} DM crude fat, 31.4–90.6 g kg^{-1} DM crude fibre, 2.4–4.6 g kg^{-1} DM phosphorus, 3.9–6.5 g kg^{-1} DM potassium and 0.98–1.41 MJ kg^{-1} DM energy (Enujiugha and Ayodele, 2003).

Information on the nature and extent of genetic variability and the degree of transmission of characters is of paramount importance in enhancing the efficiency of selection. The knowledge of correlations between various characters and their relative contribution to yield is useful for multiple character selection. This study was undertaken to assess the magnitude of genetic variability of important economic characters, the correlations between them, and their direct and indirect effects on seed yield in 'egusi' melon [*Citrullus lanatus* (Thunb.) Matsum. & Nakai].

Materials and methods

Twenty accessions of 'egusi' melon [*Citrullus lanatus* (Thunb.) Matsum. & Nakai] were used in this research. Fourteen were obtained from the germplasm of the National Institute of Horticultural Research (NIHORT), Ibadan, Nigeria and six from different towns in Nigeria. The field evaluation of these accessions was carried out at the Teaching and Research Farms, University of Agriculture, Abeokuta (7.35°N, 3.88°E 450 masl) during the early (March) and late (August) growing seasons in 2005. A double-row plot was adopted for the study in a randomized complete block design (RCBD) with three replications. Each block consisted of 40 rows and planting was done in 6 m rows with two rows of each accession. The rows were 1 m apart while the plant-to-plant distance was also 1 m. Two seeds of each accession were planted per hole and later thinned to one plant per stand. Each row therefore contained seven plants and five competitive plants within each row were observed. Manual weeding was carried out when necessary.

Data on quantitative characters were collected on ten competitive plants for each accession. These data were: days to germination, days to flowering, fruit circumference per plant, fruit weight per plant, number of branches per plant, vine length per plant, number of fruits per plant, number of seeds per fruit, seed weight per fruit, 100-seed weight, days to maturity and seed yield. Mean data were subjected to analysis of variance to estimate the variance components and coefficients of variation following Burton (1952). The broad sense heritability (h^2B) was estimated as the ratio of genotypic variance (Vg) to phenotypic variance (Vp), as described by Allard (1960). The genetic advance (GA) was estimated in accordance with the methods illustrated by Allard (1960) as $GA = K [Vp] h^2B$, where K is a constant (2.06). The genotypic and phenotypic coefficients of variation were calculated according to the procedure of Miller et al. (1958). The direct and indirect effects were calculated according to Wright (1921), Dewey and Lu (1959) and Li (1975).

Results

Generally, phenotypic variance was higher than genotypic variance (Table 1). The lowest and highest phenotypic coefficients of variation (PCV) in the early season were recorded for days to maturity (5.31) and seed yield (40.98), respectively. For the late season, PCV ranged from 5.59 for days to maturity to 55.71 for seed yield. Days to maturity showed a relatively lower PCV in both seasons. The genotypic coefficient of variation (GCV) in the early season ranged from 2.52 for fruit size to 29.62 for yield, while in the late season GCV varied from 3.50 for 100-seed weight to 46.41 for yield. Days to maturity generally had the lowest GCV value in the two seasons. Estimates of broad sense heritability ranged from 7–90% (Table 1), with seed weight per fruit having the lowest value and days to flowering and germination having the highest value.

Table 1

Range, general mean, estimate of genotypic and phenotypic variance, genotypic and phenotypic coefficient of variability, broad sense heritability and genetic advance expressed as percentage of the mean in two seasons for twenty 'egusi' melon accessions

Character	Season	Range	Mean	1	2	3	4	5	6
Days to flowering	ES	34–52	41.46	9.04	10.08	7.25	7.66	90	14.15
	LS	33.3–3.2	39.06	7.40	8.38	6.96	7.41	88	13.48
Days to germination	ES	4.0–10	6.84	1.93	2.15	20.31	21.44	90	39.64
	LS	4.0–8.8	6.13	0.82	0.97	14.77	16.07	85	27.98
Days to maturity	ES	76–101	87.53	8.78	21.6	3.39	5.31	41	4.45
	LS	80–104	90.83	12.14	25.75	3.84	5.59	47	5.43
Fruit circumference/ plant (cm)	ES	26.45–110	62.23	163.28	237.91	20.53	24.79	69	35.04
	LS	22–120.1	58.58	122.55	226.02	18.90	25.66	54	28.67
Fruit size (cm)	ES	23.01–37.68	30.69	0.60	3.56	2.52	6.15	17	2.13
	LS	25.5–47.2	36.34	5.93	12.87	6.70	9.87	46	9.37
Fruit weight/plant (g)	ES	350–3090	1012.50	43805.79	88841.96	20.67	29.44	49	29.90
	LS	250–4100	1173.17	94124.56	177771.32	26.15	35.94	53	39.20
Fruit weight (g)	ES	247–1068	546.84	6750.06	14086.49	15.02	21.70	48	21.42
	LS	200–1450	758.00	10356.14	43391.11	13.43	27.48	24	13.51
No. of branches/plant	ES	2.0–5.0	3.47	0.17	0.23	11.88	13.82	74	21.04
	LS	1.0–3.3	2.42	0.15	0.19	16.00	18.01	79	29.29
No. of fruits/plant	ES	1.0–3.6	2.02	0.13	0.19	17.85	21.58	68	30.41
	LS	1.0–3.0	1.76	0.10	0.17	17.97	23.43	59	28.39
No. of seeds/fruit	ES	53–219	141.45	199.65	619.84	9.99	17.60	32	11.68
	LS	36–442	194.38	1170.92	2676.74	17.60	26.62	44	23.99
Seed weight/fruit (g)	ES	6.45–33	17.06	12.33	22.08	20.58	27.54	56	31.68
	LS	5.74–61.44	25.92	3.23	44.4	6.93	25.71	7	3.85
100-seed weight (g)	ES	7.18–16.24	11.69	0.62	1.39	6.74	10.09	45	9.27
	LS	9.17–16.18	12.47	0.19	0.78	3.50	7.08	24	3.55
Vine length/plant (cm)	ES	130–494	236.08	2758.54	3198.47	22.25	23.96	86	42.56
	LS	126.2–390.4	258.16	1240.16	2010.53	13.64	17.37	62	22.07
Yield (kg/ha)	ES	18.01–277.90	101.79	908.83	1740.05	29.62	40.98	52	44.09
	LS	9.55–600	221.55	10570.05	15231.17	46.41	55.71	69	79.64

1: Genotypic variance; 2: Phenotypic variance; 3: Genotypic coefficient of variation (%); 4: Phenotypic coefficient of variation (%); 5: Heritability (%); 6: Genetic advance as % of the mean; ES: Early season; LS: Late season; n=60

In the early season, heritability ranged from 17% for fruit size to 90% for both days to flowering and germination, while in the late season heritability values ranged from 7% to 88% for seed weight per fruit and days to flowering, respectively. In the early season, GA ranged from 2.13% for fruit size to 44.09% for yield (Table 1), while in the late season, it ranged from 5.43% to 79.64% for days to maturity and yield, respectively.

The phenotypic correlation coefficients between 14 characters in 'egusi' melon in two seasons are presented in Table 2. In the early season, seed yield exhibited a significant negative phenotypic correlation with days to germination (–0.62), days to flowering (–0.59) and days to maturity (–0.59). However, a significant positive phenotypic correlation was observed for number of branches/plant (0.48), number of fruits/plant (0.60), fruit weight/plant (0.73) and fruit circumference/plant (0.64) during the same season. In the late season, seed yield showed a positive significant correlation with number of branches/plant (0.64), vine length/plant (0.53), number of fruits/plant (0.81), fruit circumference/plant (0.74) and number of seeds/fruit (0.60). A negative significant correlation was, however, observed for days to germination (–0.52) and days to maturity (–0.75).

Table 3 presents the genotypic correlation coefficients between 14 characters in 'egusi' melon in two seasons. In the early season, seed yield showed a significant positive correlation with number of branches/plant (0.60), number of fruits/plant (0.86), fruit weight/plant (1.28), fruit circumference/plant (0.84) and number of seeds/fruit (0.83). Days to germination (–0.83), days to flowering (–0.77) and days to maturity (–1.67) had a significant negative correlation coefficient with yield. In the late season, seed yield showed a significant positive correlation with number of branches/plant (0.77), number of fruits/plant (0.92), fruit circumference/plant (0.69) and number of seeds/fruit (1.24).

Table 2

Phenotypic correlation coefficients between 14 characters in 'egusi' melon in two seasons

Characters	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Days to germination	ES	0.52**	0.00	–0.07	–0.66**	–0.35**	–0.58**	0.14	0.14	–0.29*	–0.11	0.24	0.43**	–0.62**
	LS	–0.06	–0.36**	–0.65**	–0.66**	–0.40**	–0.67**	0.31*	0.33**	–0.14	0.06	0.30*	0.56**	–0.52**
Days to flowering	ES		–0.52**	0.63**	–0.23*	–0.38**	–0.04	0.34**	0.32*	–0.17	0.27*	0.56**	0.61**	–0.59**
	LS		–0.46**	0.39**	0.04	0.81**	0.33**	0.10	0.20	0.04	0.24	0.23	0.39**	–0.18
Number of branches/plant	ES			–0.51**	0.06	0.39**	–0.07	–0.05	–0.28*	–0.20	–0.39**	–0.15	–0.63**	0.48**
	LS			0.18	0.44**	–0.25*	0.36**	0.00	–0.25*	0.19	0.00	–0.08	–0.73**	0.64**
Vine length /plant (cm)	ES				0.28	0.09	0.54**	0.58**	0.51**	0.31*	0.60**	0.51**	0.38**	0.03
	LS				0.66**	0.71**	0.81**	0.24	0.24	0.41**	0.34**	–0.30*	–0.30*	0.53**
Number of fruits/plant	ES					0.48**	0.85**	–0.07	0.16	0.45**	0.39**	0.17	–0.31*	0.60**
	LS					0.43**	0.88**	–0.16	–0.31*	0.40**	0.25*	–0.18	–0.66**	0.81**
Fruit weight /plant (g)	ES						0.58**	0.43**	0.55**	0.30*	0.18	0.14	–0.25*	0.73**
	LS						0.69**	0.08	0.19	0.11	0.24	0.07	0.13	0.18
Fruit circum./ plant (cm)	ES							0.40**	0.43**	0.54**	0.64**	0.30*	–0.10	0.64**
	LS							–0.03	–0.11	0.35**	0.25*	–0.12	–0.46**	0.74**
Fruit circumference (cm)	ES								0.68**	0.20	0.48**	0.44**	0.38**	0.24
	LS								0.90**	0.43**	0.52**	–0.27*	0.22	0.06
Fruit weight (g)	ES									0.28*	0.55**	0.40**	0.58**	0.06
	LS									0.38**	0.52**	–0.19	0.40**	–0.06
Number of seeds/fruit	ES										0.73**	0.15	0.05	0.47**
	LS										0.90**	–0.28*	–0.29*	0.60**
Seed weight/ fruit (g)	ES											0.52**	0.42**	0.21
	LS											–0.07	–0.03	0.41**
100-seed weight (g)	ES												0.39**	0.02
	LS												0.39**	–0.35**
Days to maturity	ES													–0.59**
	LS													–0.75**

1: Season; 2: Days to flowering; 3: Number of branches/plant; 4: Vine length/plant (cm); 5: Number of fruits/plant; 6: Fruit weight/plant (g); 7: Fruit circumference/plant (cm); 8: Fruit circumference (cm); 9: Fruit weight (g); 10: Number of seeds/fruit; 11: Seed weight/fruit (g); 12: 100-seed weight (g); 13: Days to maturity; 14: Seed yield (kg/ha); ES: Early season; LS: Late season; n = 60; *, ** Significant at the 5% and 1% level of probability, respectively

Table 3

Genotypic correlation coefficients between 14 characters in 'egusi' melon in two seasons

Characters	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Days to germination	ES	0.58**	0.02	–0.07	–0.79**	–0.51**	–0.68**	0.23	0.19	–0.45**	–0.12	0.40**	0.74**	–0.83**
	LS	–0.09	–0.43**	–0.79**	–0.85**	–0.51**	–0.85**	0.50**	0.79**	–0.22	0.33*	0.77**	0.79**	–0.61**
Days to flowering	ES		–0.59**	0.74**	–0.27*	–0.50**	–0.01	1.17**	0.59**	–0.21	0.41**	0.88**	1.02**	–0.77**
	LS		–0.55**	0.53**	0.05	1.20**	0.44**	0.22	0.54**	0.16	1.11**	0.55**	0.56**	–0.23
Number of branches/plant	ES			–0.65**	–0.06	0.38**	–0.24	–0.62**	–0.67**	–0.58**	–0.66**	–0.27*	–1.22**	0.60**
	LS			0.24	0.60**	–0.54**	0.46**	–0.03	–0.58**	0.30*	–0.10	–0.16	–1.14**	0.77**
Vine length /plant (cm)	ES				0.26*	0.07	0.62**	1.46**	0.74**	0.52**	0.83**	0.76**	0.66**	–0.02
	LS				0.72**	0.93**	0.87**	0.48**	0.72**	0.96**	2.20**	–0.56**	–0.20	0.47**
Number of fruits/plant	ES					0.38**	0.87**	–0.27*	0.14	0.72**	0.52**	0.29*	–0.54**	0.86**
	LS					0.50**	0.92**	–0.27*	–0.88**	0.78**	1.47**	0.11	–0.99**	0.92**
Fruit weight /plant (g)	ES						0.49**	0.50**	0.39**	0.36**	0.06	0.05	–0.52**	1.28**
	LS						0.79**	0.25*	0.62**	0.55**	2.13**	–0.06	0.51**	–0.09
Fruit circum./ plant (cm)	ES							0.79**	0.48**	0.89**	0.80**	0.50**	–0.27*	0.84**
	LS							–0.04	–0.29*	0.85**	1.81**	0.06	–0.52**	0.69**
Fruit circumference (cm)	ES								0.70**	–0.29*	0.74**	0.91**	1.49**	0.39**
	LS								0.94**	0.02	–0.48**	–0.77**	0.24	0.19
Fruit weight (g)	ES									0.30*	0.69**	0.61**	1.43**	–0.04
	LS									–0.37**	–1.68**	–0.60**	0.80**	0.00
Number of seeds/fruit	ES										0.67**	–0.09	0.32*	0.83**
	LS										1.35**	–0.57**	–0.79**	1.24**
Seed weight/ fruit (g)	ES											0.60**	0.95**	0.14
	LS											–0.20	–0.71**	2.45**
100-seed weight (g)	ES												0.49**	–0.21
	LS												0.99**	–0.75**
Days to maturity	ES													–1.67**
	LS													–0.96**

1: Season; 2: Days to flowering; 3: Number of branches/plant; 4: Vine length/plant (cm); 5: Number of fruits/plant; 6: Fruit weight/plant (g); 7: Fruit circumference/plant (cm); 8: Fruit circumference (cm); 9: Fruit weight (g); 10: Number of seeds/fruit; 11: Seed weight/fruit (g); 12: 100-seed weight (g); 13: Days to maturity; 14: Seed yield (kg/ha); ES: Early season; LS: Late season; n = 60; *, ** Significant at the 5% and 1% level of probability, respectively

The environmental correlation coefficients between 20 'egusi' melon accessions in two seasons are presented in Table 4. In the early season, there was no significant correlation between seed yield and the characters studied. During the late season, seed yield showed a significant correlation with fruit circumference/plant (0.83), fruit weight/plant (0.63) and vine length/plant (0.66).

The direct and indirect effects of various characters on seed yield, as well as their residual effects during the early and late seasons, are given in Table 5. In the early season, vine length had the largest positive direct effect on seed yield (5.76), followed by number of fruits/plant (1.90) and number of branches/plant (1.24). Days to flowering, however, had the largest negative direct effect (–4.09). The residual effect was 0.39. During the late season, fruit circumference/plant had the largest direct effect (2.21), followed by number of seeds/fruit (1.29). The largest negative direct effect on seed yield was observed for days to flowering (–1.05), though its correlation was not significant. The residual effect for the late season was 0.44.

Table 4
Environmental correlation coefficients between 14 characters in 'egusi' melon in two seasons

Characters	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Days to germination	ES	0.03	–0.12	–0.08	–0.21	–0.05	–0.22	0.17	0.04	–0.18	–0.14	–0.04	–0.09	–0.23
	LS	0.12	0.00	–0.31*	–0.24	–0.23	–0.35**	0.01	–0.08	–0.04	–0.06	–0.15	0.20	–0.24
Days to flowering	ES		–0.22	–0.19	–0.14	–0.21	–0.17	–0.38**	–0.28*	–0.21	–0.08	0.01	–0.03	–0.29*
	LS		0.04	0.00	0.01	–0.04	0.11	–0.18	–0.15	–0.25*	–0.13	–0.10	0.12	0.04
Number of branches/plant	ES			0.09	0.36**	0.40**	0.34**	0.35**	0.33**	0.20	0.11	0.02	0.12	0.29*
	LS			0.06	0.09	0.35**	0.21	0.06	0.02	0.02	0.05	–0.02	–0.09	0.26*
Vine length /plant (cm)	ES				0.35**	0.15	0.28*	0.07	0.13	0.13	0.11	0.13	–0.03	0.18
	LS				0.58**	0.43**	0.74**	–0.05	–0.06	–0.18	–0.21	–0.16	–0.43**	0.66**
Number of fruits/plant	ES					0.65**	0.82**	0.05	0.21	0.23	0.17	0.01	–0.06	0.22
	LS					0.35**	0.86**	–0.04	0.03	0.00	–0.09	–0.41**	–0.31*	0.65**
Fruit weight /plant (g)	ES						0.74**	0.44**	0.70**	0.27*	0.32*	0.22	–0.04	0.16
	LS						0.59**	–0.10	–0.06	–0.29*	–0.27*	0.15	–0.24	0.63**
Fruit circum./ plant (cm))	ES							0.26*	0.39**	0.27*	0.39**	0.07	0.11	0.36**
	LS							–0.03	–0.01	–0.12	–0.16	–0.24	–0.39**	0.83**
Fruit circumference (cm)	ES								0.74**	0.35**	0.41**	0.28*	–0.01	0.19
	LS								0.91**	0.76**	0.86**	–0.03	0.21	–0.10
Fruit weight (g)	ES									0.27*	0.41**	0.23	–0.10	0.16
	LS									0.76**	0.88**	–0.06	0.21	–0.13
Number of seeds/fruit	ES										0.82**	0.30*	–0.11	0.22
	LS										0.92**	–0.15	0.13	–0.21
Seed weight/ fruit (g)	ES											0.44**	–0.06	0.30*
	LS											–0.05	0.14	–0.27*
100-seed weight (g)	ES												0.32*	0.23
	LS												0.08	–0.07
Days to maturity	ES													0.34*
	LS													–0.49**

1: Season; 2: Days to flowering; 3: Number of branches/plant; 4: Vine length/plant (cm); 5: Number of fruits/plant; 6: Fruit weight/plant (g); 7: Fruit circumference/plant (cm); 8: Fruit circumference (cm); 9: Fruit weight (g); 10: Number of seeds/fruit; 11: Seed weight/fruit (g); 12: 100-seed weight (g); 13: Days to maturity; 14: Seed yield (kg/ha); ES: Early season; LS: Late season; n = 60; *, ** Significant at the 5% and 1% level of probability, respectively

Discussion

Significant differences were observed between the accessions for most of the characters, indicating that there is sufficient variability available for effective selection. The phenotypic coefficient of variation (PCV) was generally higher than the genotypic coefficient of variation (GCV) for all the characters across the two growing seasons and in many cases the two values only differed slightly, indicating that environmental factors influenced their expression to some degree.

The PCV values obtained implied greater genetic variability between the accessions and the responsiveness of the characters for making further improvements by selection. Generally, the higher heritability estimates for days to germination and flowering, fruit circumference per plant, number of branches per plant and vine length per plant indicated that environmental factors did not greatly affect the phenotypic variation of these characters, selection for which on the basis of phenotypic performance was likely to be dependable and effective. The relatively low heritability estimates for days to maturity, fruit circumference and fruit weight suggest the ineffectiveness of direct selection for such characters. The moderate to high values of heritability, GCV and GA observed for days to germination, fruit circumference per plant, fruit weight per plant, number of branches per plant, number of fruits per plant, seed weight per fruit and vine length per plant could be attributed to additive gene action, thus making selection for them simple. However, the moderate estimates of heritability coupled with low GCV and GA observed for 100-seed weight and days to flowering suggest that these characters were governed by non-additive gene action coupled with high genotype–environment interaction. The heritability observed might be due to the favourable influence of the environment rather than the genotype, and simple selection will not be rewarding. However, these characters could be improved by the development of hybrid varieties or the isolation of transgressive segregants in heterosis. The estimates of phenotypic and genotypic correlation coefficients revealed that genotypic correlation was higher than the corresponding values for all the characters studied, indicating that the inherent association between the characters is governed largely by genetic causes, although it could also be affected by environmental forces.

Table 5

Direct and indirect effects of various characters on seed yield in 'egusi' melon

Characters	Season	1	2	3	4	5	6	7	8	9	10
Days to flowering	ES	–4.09		–0.73	4.26	–0.51	0.22	0.04	0.03	0.02	–0.77**
	LS	–1.05		0.48	–0.26	–0.02	0.01	0.97	0.21	–0.57	–0.23
Number of branches/plant	ES	1.24	2.41		–3.74	–0.11	–0.16	0.91	0.09	–0.03	0.60**
	LS	–0.88	0.58		–0.12	–0.26	–0.01	1.02	0.39	0.05	0.77**
Vine length/plant (cm)	ES	5.76	–3.03	–0.81		0.49	–0.03	–2.36	–0.08	0.04	–0.02
	LS	–0.49	–0.56	–0.21		–0.32	0.01	1.92	1.24	–1.12	0.47**
Number of fruits/plant	ES	1.90	1.10	–0.07	1.50		–0.16	–3.31	–0.11	0.03	0.86**
	LS	–0.44	–0.05	–0.53	–0.35		0.01	2.03	1.01	–0.75	0.92**
Fruit weight/ plant (g)	ES	–0.43	2.05	0.47	0.40	0.72		–1.87	–0.05	0.00	1.28**
	LS	0.01	–1.26	0.48	–0.46	–0.22		1.75	0.71	–1.09	–0.09
Fruit circumference/ plant (cm)	ES	–3.81	0.04	–0.30	3.57	1.65	–0.21		–0.13	0.04	0.84**
	LS	2.21	–0.46	–0.40	–0.43	–0.40	0.01		1.10	–0.92	0.69**
Number of seeds/fruit	ES	–0.15	0.86	–0.72	3.00	1.37	–0.15	–3.39		0.03	0.83**
	LS	1.29	–0.17	–0.26	–0.47	–0.34	0.01	1.88		–0.69	1.24**
Seed weight/ fruit (g)	ES	0.05	–1.68	–0.82	4.78	0.99	–0.03	–3.05	–0.10		0.14
	LS	–0.51	–1.17	0.09	–1.08	–0.65	0.02	4.00	1.74		2.45**

1: Direct effect on seed yield; 2: Days to flowering; 3: Number of branches/plant; 4: Vine length/plant (cm); 5: Number of fruits/plant; 6: Fruit weight/plant (g); 7: Fruit circumference/plant (cm); 8: Number of seeds/fruit; 9: Seed weight/fruit (g); 10: Genotypic correlation coefficients; Residual effects: ES = 0.39; LS - 0.44; ES: Early season; LS: Late season; *, ** Significant at the 5% and 1% level of probability, respectively

In the early season, significant phenotypic correlation was observed between seed yield and number of branches per plant, number of fruits per plant, fruit weight per plant and fruit circumference per plant, while in the late season, significant phenotypic correlation was observed between number of branches per plant, vine length per plant, number of fruits per plant, fruit circumference per plant and seed yield. This suggests that these characters possessed greater practical value for selection than the other component characters.

Characters which are phenotypically correlated but not genotypically correlated would not produce repeatable estimates of inter-character associations and any selection based on such relationships is likely to be unreliable. Those that are phenotypically and genotypically correlated with seed yield would produce repeatable estimates of inter-character association and only selection based on their relationship would result in a significant improvement in seed yield in ‘egusi’ melon [*Citrullus lanatus* (Thunb.) Matsum. & Nakai].

The significant genotypic and phenotypic correlation between days to maturity and days to flowering implied that the number of days to flowering can be used as a criterion for selecting lines with a short life span by selecting lower number of days to flowering. Early flowering lines which flower early will be suited for areas with a short growing season. The significant positive correlation between vine length per plant and number of fruits per plant implies that plants with longer vines produce more fruits because of the increased number of fruit-bearing nodes, the greater photosynthetic area and the consequently higher seed yield, as observed in the early season.

During the late season, the significant genotypic and phenotypic correlation between seed yield and number of branches per plant, vine length per plant, number of fruits per plant and fruit circumference per plant suggests that selection based on phenotypic performance will be beneficial.

The significant phenotypic and environmental correlation between seed yield and vine length per plant, number of fruits per plant, fruit weight per plant and fruit circumference per plant showed the ineffectiveness of direct selection for seed yield via these characters as they were profoundly affected by environmental factors.

Correlation analysis measures mutual association, with no regard to causation, whereas path analysis specifies causes and measures their relative importance (Dewey and Lu, 1959). Despite the strong positive association of number of seeds per fruit with seed yield, its direct effect was negative, thus indicating the inefficiency of selection based on correlation alone. The number of branches per plant, vine length per plant and seed weight per fruit can be used for direct selection to improve seed yield in ‘egusi’ melon. Vine length per plant had a positive contribution to seed yield despite its non-significant correlation. This demonstrates the limitation of selecting on the basis of inter-correlation, as this may not produce the desired result.

The residual effect of 0.39 for the early season and 0.44 for the late season implied that 61% and 56% of the total variation in seed yield in the early and late seasons, respectively, had been determined. It further portrayed the existence of other factors, not considered in this study, which may contribute to seed yield in 'egusi' melon.

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Review

PHYSIOLOGICAL STATUS OF CULTIVATED PLANTS CHARACTERISED BY MULTI-WAVELENGTH FLUORESCENCE IMAGING

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The fluorescence imaging technique was elaborated primarily for the detection of the fluorescence traits accompanying changes in the physiological status of stressed plants. The paper summarises the conditions and technical background required for the use of multi-wavelength fluorescence imaging. Images of leaves were recorded at wavelengths of 440, 520, 690 and 740 nm. Possible applications are illustrated by studies on the leaves of stressed plants. An evaluation of the images is presented, including the necessary corrections and fluorescence ratios, examples of comparisons between imaging and functional activity measurements, and an evaluation of the diagnostic importance and reliability of imaging in detecting the effects of stressors in plants. The results demonstrate that the multi-wavelength fluorescence imaging of leaves is a useful method for detecting the presence of stress in plants and for determining the extent of the stress.

Key words: biotic stress, blue-green fluorescence, fluorescence imaging, plant stress detection

Introduction

Fluorescence characteristics of plants

Many of the compounds found in plants are capable of fluorescing when subjected to excitation at a certain wavelength. In response to UV excitation leaves have an emission maximum between 440 and 460 nm (F440) in the blue spectral range and exhibit a shoulder to varying extents in the green range around 520–530 nm (F520) (Chappelle et al., 1984; Stober and Lichtenthaler, 1993). In green leaves this blue and green fluorescence originates primarily from chlorophyll-free epidermis cells and from the largest leaf veins and cell walls (Stober and Lichtenthaler, 1993; Lang and Lichtenthaler, 1994). Little or no blue and green fluorescence emission is observed in the mesophyll cells, as this is absorbed partially or completely by the photosynthetic pigments of the cells

(Stober and Lichtenthaler, 1993). Reabsorption is less characteristic of green fluorescence, so compared to blue fluorescence it is more intensive in the region between the veins than in the vascular bundles. However, it is rarely more intense than the blue fluorescence (Lichtenthaler et al., 1996).

Investigations carried out by Schweiger (1999) on 21 different plant species demonstrated that the ferulic acid esterified to cell-wall polysaccharides was a significant emitter of blue fluorescence, while other hydroxycinnamic acid derivatives in the cell-wall, such as p-coumaric acid and caffeic acid, only made a negligible contribution to blue fluorescence. Kaempferol and quercetin glycosides fluoresce in the green spectral range (Schweiger, 1999), as do berberin and riboflavin (Lang et al., 1991). The level of green fluorescence is also influenced by reabsorption and subsequent reemission by carotenoids (Lichtenthaler, 1995).

If UV irradiation is not absorbed in the epidermis, but reaches the parenchyma cells, it excites the red (F690) and far-red (F740) fluorescence of chlorophyll-a, particularly in the upper chloroplast layer of the palisade parenchyma cells, immediately below the epidermis. Plants grown in the laboratory or greenhouse, where the illumination is generally less intense and poorer in UV rays than under field conditions, exhibit relatively intensive UV-induced chlorophyll-a fluorescence, but this progressively declines as the intensity of the light available for plant growth rises (Stober and Lichtenthaler, 1993). Plants grown in the open under full sunlight and natural UV irradiation contain many times more soluble flavonoids (flavones, flavonols, chalcones) and cinnamic acid derivatives than greenhouse plants (Lichtenthaler and Schweiger, 1998). In such cases the majority of the UV irradiation reaching the plants is absorbed by the epidermal cells, so very little enters the mesophyll. Consequently, field-grown plants have relatively little red or far-red fluorescence originating from chlorophyll-a. In green leaves the chlorophyll fluorescence arises from the intercostal areas.

The level of blue and/or green fluorescence and how it compares to the red chlorophyll fluorescence differs from one species to another. In monocotyledons the quantity of phenols bound to the cell-wall is much higher than in dicotyledons (Harris and Hartley, 1976; Schweiger, 1999), so monocots, such as grasses and sedges, emit much more intensive blue and green fluorescence (Chappelle et al., 1985; Stober and Lichtenthaler, 1993; Johnson et al., 2000). The intensity of fluorescence depends on the concentration of the emitting compound, on leaf traits influencing spectral traits (e.g. cell arrangements, intercellular spaces), on the reabsorption of emitted fluorescence, and, in the case of red and far-red fluorescence, on the energy distribution between photosynthesis, heat emission and chlorophyll fluorescence (Buschmann et al., 2000). There is considerable variability in the intensity of fluorescence bands, both within a single plant (Lang et al., 1991) and between species.

Fluorescence in the leaves of stressed plants

In the course of growth, plants are exposed to numerous biotic and abiotic stress factors. The biotic stressors are primarily pathogenic microorganisms, such as viruses, bacteria and fungi, but damage due to feeding by insects and snails also comes under this category, while the abiotic stressors include factors causing extreme changes in the plant environment, such as heat, frost, drought, strong light or UV irradiation, nutrient deficiency, toxic heavy metals, etc. Stressors in both groups generally induce defensive responses leading to better adaptation, but requiring surplus energy and the synthesis of various compounds, thus causing profound changes in the plant metabolism. Depending on the duration of the stress effect, these may cause yield losses and/or a decline in quality in the case of cultivated crops.

In the course of the responses to various stressors, metabolites with characteristic fluorescence properties accumulate in the plants. These are compounds emitting fluorescence in the blue and green spectral ranges. Directly or indirectly the stressors may modify or damage the photosynthetic apparatus, thus causing changes in the fluorescence properties of the most important photosynthetic pigment, chlorophyll-a. Fluorescence imaging, especially in the red and far-red regions, is widely used to trace the functioning of the photosynthetic apparatus. Using techniques specially devised for this purpose, parameters characteristic of photosynthetic functioning, such as Fv/Fm, photochemical quenching and non-photochemical quenching (Genty and Meyer, 1994; Baker et al., 2001; Nedbal and Whitmarsh, 2004; Ralph et al., 2005) and Rfd [(Fm–Fs)/Fs] (Lichtenthaler et al., 2005), can be directly imaged. In addition to these parameters, important information can also be obtained on the quantity of fluorescence emitted in the blue and green spectral regions and on how it compares with that emitted in the red and far-red region, information which is extremely useful for stress physiological analysis. The multi-wavelength fluorescence imaging system is ideal for the investigation of these aspects of fluorescence.

Certain plant stressors, such as nitrogen deficiency or a great surplus of light, reduce the red fluorescence of chlorophyll-a, while the intensity of the blue and green fluorescence may remain unchanged, or even increase (Stober and Lichtenthaler, 1993; Langsdorf et al., 2000). This rise in the intensity of blue and green fluorescence is due in part to the slow decline in the quantity of pigments absorbing blue and green fluorescence (chlorophylls and carotenoids) and also to the higher epidermal concentration of compounds that absorb UV and fluoresce in the blue and green ranges. As a consequence less UV radiation reaches the mesophyll cells, again resulting in a reduction in red fluorescence (Schweiger et al., 1996). Some stressors may also aggravate chlorophyll destruction or retard biosynthesis; the lower self-absorption due to the decreased chlorophyll content then results in more intensive red fluorescence at 690 nm and in an increase in the F690/F740 ratio, which is an inverse indicator of the *in situ* chlorophyll content (Lichtenthaler et al., 1990; Subhash et al., 1999; Gitelson et al., 1999). Changes in physiological status are thus clearly reflected in the various fluorescence ratios (F440/F690, F440/F740, F690/F740).

In tobacco plants treated with diuron, a herbicide that inhibits photosynthesis, a reduction was observed in the F440/F690 and F690/F740 ratios, since the chlorophyll fluorescence increased due to the inhibition of photosynthesis, while there was no significant change in the intensity of blue fluorescence (Lichtenthaler et al., 1997). Similar changes were reported in heat-treated tobacco (Lang et al., 1996). Stressors generally cause less fluctuation in the intensity of blue fluorescence than in that of chlorophyll fluorescence (Lichtenthaler and Rinderle, 1988; Stober and Lichtenthaler, 1993), so the F440/F690 and F440/F740 ratios are good early indicators of stress, responding sensitively to changes in environmental conditions and giving a good reflection of altered photosynthetic functions (Heisel et al., 1996; Buschmann and Lichtenthaler, 1998; Langsdorf et al., 2000).

As noted above, these ratios are also influenced by the reabsorption of blue and green fluorescence; that of green fluorescence is considerably smaller than that of blue fluorescence, as chlorophyll-a does not absorb in this spectral region, so there may be a reduction in the F440/F520 fluorescence ratio when the chlorophyll content declines (Lichtenthaler et al., 1996). This ratio is relatively stable in the case of short-term stress, but in the long term there is often an increase in the emission of green fluorescence (Lang et al., 1996; Buschmann and Lichtenthaler, 1998). Certain pathogens, such as phytopathogenic fungi, also emit fluorescence, which increases the intensity of the blue-green fluorescence (Lüdecker et al., 1996). A good example of this is the fluorescence image of vine leaves infected with powdery mildew, where the site of infection exhibits intensive blue fluorescence (Szigeti, unpublished result).

The type of stress and the nature of plant responses determine which fluorescence ratios decrease and which increase. The changes observed in fluorescence ratios in response to stressors are presented in Table 1.

Table 1
Changes in the blue/red (F440/F690), blue/far-red (F440/F740), red/far-red (F690/F740) and blue/green (F440/F520) fluorescence ratios as stress indicators in leaves
(Buschmann et al., 2000 with additional data)

Stressor	F440/F690	F440/F740	F690/F740	F440/F520
Water deficiency	+ +	+ +	0	0
N deficiency	+ +	+ +	+	0
Strong light	+ +	+ +	+	– –
Mite attack	+ +	+ +	0	+
Rust infection	+ +	+ +	+ +	–
Heat stress	– –	– –	0	–
UV-A stress	– –	– –	0	+
Photoinhibition	+ +	+ +	– –	0
Paraquat treatment	+	+	–	–
Cd stress	+	+	+	–
Diuron treatment	– –	– –	+	0

++ = significantly higher, + = higher, – – = significantly lower, – = lower, 0 = no significant change

Imaging of whole leaves versus measurements on small surfaces

The determination of most physiological parameters (absorption or fluorescence spectrum, quantitative determination of pigments or other compounds, gas exchange measurements) is carried out using point measurements on small leaf surfaces or using small leaf samples. However, the functional changes induced by stressors are not of the same extent or character either on all the leaves of a given plant, or on leaf parts of different ages and in different stages of development. In multispectral fluorescence imaging, the blue and green fluorescence from the cell walls and the red and far-red chlorophyll fluorescence give the best early detection of stress symptoms in plants. Current imaging techniques allow pre-symptomatic changes in the physiological status to be monitored non-destructively. Thermal, reflectance and fluorescence imaging have proved their potential by detecting stress-related changes in the pattern of light emission from plant leaves (Chaerle and Van der Straeten, 2000; 2001). By imaging chlorophyll fluorescence, it is possible to produce parameterised fluorescence images that estimate the operating quantum efficiency of photosystem II photochemistry and which can be used to reveal heterogeneous patterns of photosynthetic performance within leaves (Baker et al., 2001). In the light of the above, the accuracy and reliability of determining physiological parameters on the basis of point measurements can be improved by determining the distribution of certain functional traits over the leaf surface. This is of particular significance in the early stages of stress or pathogen infection, when the whole leaf surface is rarely affected at first, but the symptoms spread gradually through the leaf with the pathogen (virus, bacterium, fungus). The heterogeneity of the symptoms can thus be depicted.

Brief outline of how the fluorescence imaging system works

The fluorescence imaging system used in our department was elaborated in cooperation with German, French and Hungarian colleagues within the framework of an INCO Copernicus project and was tested for use in measuring plant samples in the department. A sketch of the system is provided in Figure 1.

In the fluorescence imaging system, the excitation light source inducing the fluorescence of the plant sample is a 16.7 Hz xenon flash lamp, delivering light with a wavelength of 350 nm through a DUG 11 filter (Schott, Jena, Germany). This wavelength is a technical compromise, as it allows both blue/green and red/far-red fluorescence to be induced simultaneously.

The fluorescence emitted by the sample reaches the CCD camera (resolution: ½ megapixel), which is synchronised with the flash of the xenon lamp, through an optical image intensifying system. The filters incorporated into the camera ensure that only the wavelength to be tested reaches the camera, which can thus produce complete images of the sample at 440, 520, 690 and 740 nm. The intensification of low intensity fluorescence signals is achieved by image accumulation, involving several hundred or even several thousand images, depending on the intensity of the fluorescence. Images at each wavelength are obtained by carrying out consecutive measurements. The camera is computer-controlled, allowing image correction and analysis, and the calculation of ratios, differences, etc. In the course of evaluation, the software corrected the images based on the spectral sensitivity of the filters. For a detailed description of the method, see Lichtenthaler and Babani (2000) and Lenk and Buschmann (2006).

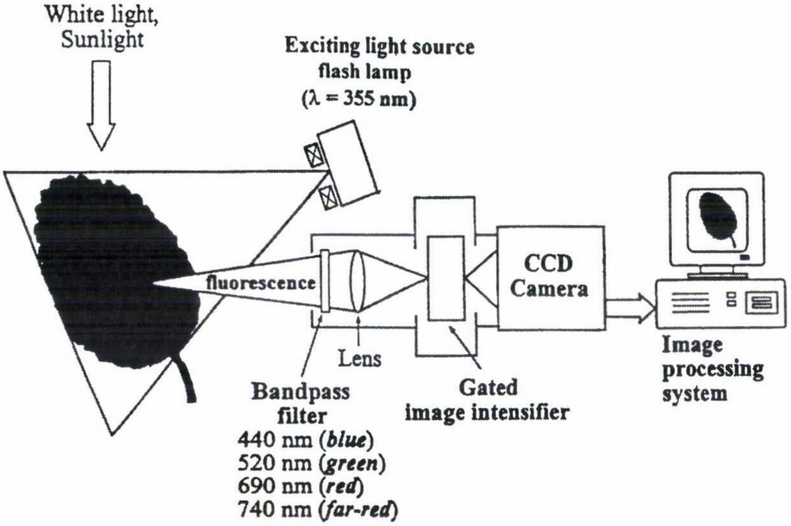

Fig. 1. Sketch of the fluorescence imaging system (FIS)

A number of technical problems, such as differences in leaf thickness or the presence of prominent veins or crinkles, make it difficult to obtain high-quality images. For accurate measurements it is essential for the leaf surface to be illuminated uniformly by the UV excitation light. This could not be achieved perfectly with the instrument previously available, since the angle of incidence of the exciting light was not identical to the detection angle, which was 90° to the plane of the sample. By means of satisfactory correction, however, the differences in fluorescence intensity arising from this non-uniformity in the illumination could be eliminated. This correction involved adding the inverse of the 440 nm fluorescence emission of an illuminated sheet of white paper to the fluorescence images recorded at various wavelengths. It is also important to check regularly that the non-fluorescent light reaching the detector and the internal noise of the camera are filtered out, and that the lamp and the camera are properly synchronised. The latter is extremely important, as the camera works in impulse mode, only opening when the xenon lamp emits a flash. The flash and the camera are synchronised by means of an antenna that senses the electromagnetic field generated in the power supply cable of the lamp by the very strong current (850–900 amperes). This innovation was developed by the staff of the Atomic Physics Department at Budapest University of Technology and Economics, who also elaborated a system whereby excitation is achieved not by a flash lamp, but by short-wavelength blue LEDs placed concentrically around the camera, ensuring more uniform illumination (Kocsányi et al., unpublished data).

Examples of how fluorescence imaging can be applied

The use of fluorescence imaging to detect the effect of a biotic stressor will be demonstrated on detached leaves of pot-grown tobacco (*Nicotiana tabacum* cv. Samsun) plants infected with Obuda pepper virus (ObPV). In susceptible plants, virus infection often induces mosaic, chlorotic spots on infected leaves. These pathological changes are often associated with a reduction in photosynthetic activity, but functional activity may be impaired even in leaf sections that are apparently free of symptoms.

As can be seen in Figure 2, in untreated control leaves the blue and green fluorescence was most intense along the leaf veins, while the red and far-red fluorescence arising from chlorophyll-a was more intense in the intercostal region (between the veins). It is also clear from the figure that the blue and green fluorescence had lower intensity than the red and far-red (note the difference in the intensity scale). In virus-infected leaves, on the other hand, although there was an increase in the blue and green fluorescence around the veins compared with the untreated control (Fig. 3), the red and far-red fluorescence was an order of magnitude more intense than the blue. Compared with untreated control leaves, there was an increase of at least four to eight times in the red and far-red spectral region. Evaluation is more precise if characteristic ratios are compared, rather than the numerical values of the intensity of each wavelength. The intensity ratios calculated from corrected images of the fluorescence emitted by the leaves are illustrated in Figure 4. It is clear from the figure that the F440/F520, F440/F690 and F440/F740 ratios decreased due to the stress induced by virus infection, while the F690/F740 ratio reflecting changes in chlorophyll-a fluorescence increased. These ratio changes demonstrated that virus infection caused a greater increase in the quantity of compounds fluorescing at 520 nm than in those fluorescing at 440 nm. In other words, virus infection in tobacco leaves enhanced the quantity of compounds emitting fluorescence in the green spectral region; it is also possible, however, that the self-absorption of blue fluorescence increased (Lichtenthaler et al., 1996). These changes could be detected even when there were no visible symptoms, as recently demonstrated in leaves infected with tobacco mosaic virus (Chaerle et al., 2007; Pineda et al., 2008). Using a confocal laser scanning microscope, the distribution of blue fluorophores within the leaf tissue was also examined in barley plants (Hideg et al., 2002).

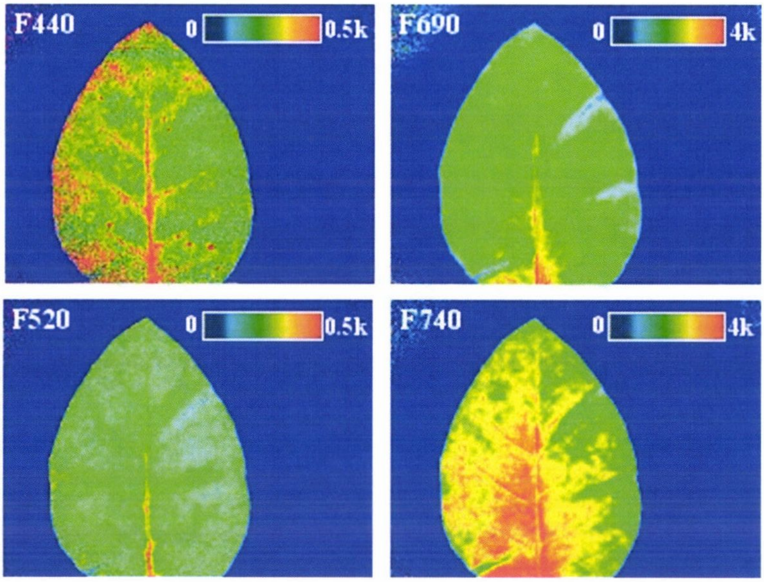

Fig. 2. Fluorescence images of untreated control tobacco (*Nicotiana tabacum* cv. Samsun) leaves taken in the blue (440 nm), green (520 nm), red (690 nm) and far-red (740 nm) spectral regions. The colours are false colours indicating the intensity of the emitted fluorescence: blue < green < yellow < red

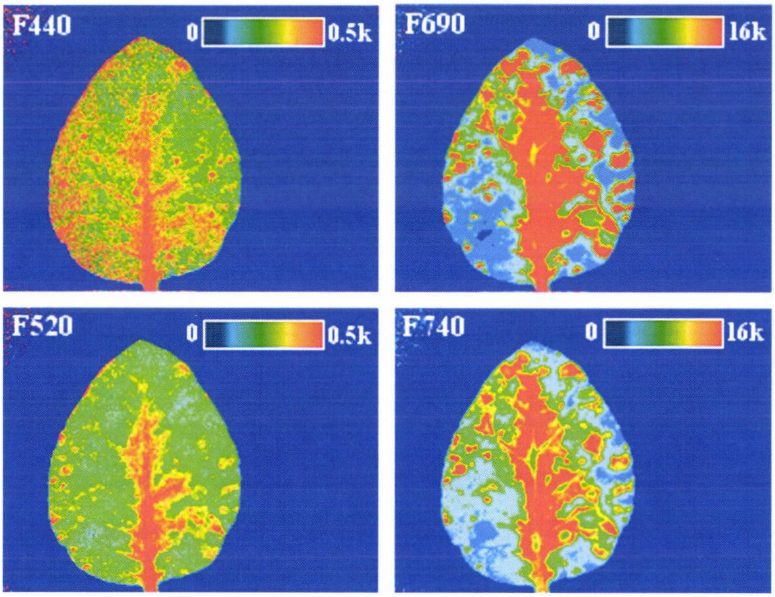

Fig. 3. Fluorescence images of tobacco (*Nicotiana tabacum* cv. Samsun) leaves treated with *Obuda pepper virus* (ObPV) taken in the blue (440 nm), green (520 nm), red (690 nm) and far-red (740 nm) spectral regions. The colours are false colours indicating the intensity of the emitted fluorescence: blue < green < yellow < red

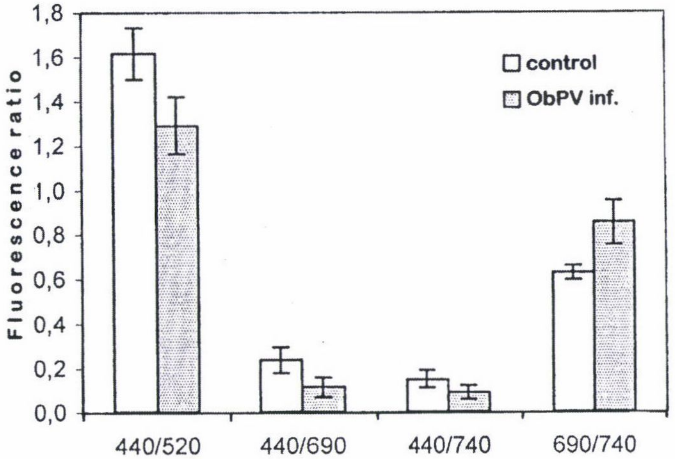

Fig. 4. Fluorescence ratios F440/F520, F440/F690, F440/F740 and F690/F740 calculated for the untreated control and for tobacco (*Nicotiana tabacum* cv. Samsun) leaves treated with *Obuda* pepper virus (ObPV)

The photosynthetic parameters of the untreated control and of tobacco leaves infected with ObPV were recorded in order to determine what changes in functional activity were associated with the fluorescence changes outlined above (Table 2).

As can be seen from the table, there was a 40% reduction in the chlorophyll content, while the chlorophyll a/b ratio dropped from 3.52 to 3.17, suggesting that chlorophyll-a decomposed to a greater extent in response to virus infection than chlorophyll-b. This was associated with a decrease of around 40% in the CO_2 fixation characteristic of the functioning of the whole photosynthetic apparatus. The inhibition of CO_2 fixation is caused partly by the inhibition of photosynthetic electron transport, since there was also a decline in the ratio of variable to maximum fluorescence (Fv/Fm) indicative of the photochemical quantum efficiency of PS2. There was a corresponding change in the fluorescence decay time, indicating the retardation of electron transport between the Q_A and Q_B components of the photosynthetic electron transport chain. Based on a large number of experiments involving various plants and stressors, certain correlations could be detected between changes in functional parameters and in the fluorescence intensity ratios or fluorescence differences calculated from the images. It would appear that multi-wavelength fluorescence imaging will complement rather than replace the measurement of functional activities, since it provides information on the distribution of the symptoms over the whole leaf. Imaging can be used for the separate evaluation of leaf sections with non-average properties.

Table 2

Photosynthetic functional characteristics of the untreated control and of tobacco (*Nicotiana tabacum* cv. Samsun) leaves treated with *Obuda pepper virus* (ObPV)

Sample	Chlorophyll a+b (μg/g fr. w.)	Chl a/b	CO_2 fixation (%)	Fluor. decay time $t_{1/2}$ (μs)	F_v/F_m
Control	1522 ± 188	3.52 ± 0.12	100	259 ± 9	0.82 ± 0.03
ObPV	899 ± 244	3.17 ± 0.32	58	314 ± 12	0.63 ± 0.10

Certain compounds, such as S-methylmethionine, are well-known to provide protection against stress (for review, see Szegő et al., 2007). Using fluorescence imaging, this protective effect could be clearly detected in 4–6-week-old maize plants (Szigeti, unpublished data). A further example of the many uses of multi-wavelength fluorescence imaging is the detection of UV sensitivity in maize lines (Pintér et al., 2007). All these examples prove that the multi-wavelength fluorescence imaging of leaves is a satisfactory laboratory method for detecting both the presence of stress and the level of stress suffered by plants.

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Review

ECOLOGICAL PROBLEMS IN THE CARPATHIAN MOUNTAINS AND POSSIBLE WAYS TO OVERCOME THEM

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Introduction

In recent decades, mankind has become ever more concerned about environmental problems. We seem to have finally comprehended that the environment is the space where we must all live and bring up our children rather than just an abstract notion. People have started to take active care of soils, water and the atmosphere and to worry about the preservation of biodiversity. However, these efforts are sometimes insufficient when a country shows concern about its own environment, while neglecting the ecological problems of their neighbours. It is like tidying up one room without trying to look after the whole apartment. Nature acknowledges no state borders or other boundaries, but state borders, including those with the EU, have a negative environmental effect, reflected in the acute ecological problems currently faced (Antosiak et al., 1997; Didukh, 2004; Mezherin et al., 1991; Nikolaichuk, 2002). The problems of preserving the natural diversity of the Carpathian Mountains have been dealt with in numerous scientific theses and are the basis of the practical generalizations put forward in this paper.

For Ukraine, environmental problems are especially important, as most of the ecological and social indices in the country are far worse than those in developed countries and in Europe as a whole. In particular, Ukraine has the largest percentage of arable land (55%) of any country in Europe. This figure is 2.5 times higher than the European average, while the area of nature reserves is 2.5 times smaller than the European average. Ukraine has the smallest per capita area of natural flora (0.35 ha, of which forests make up only 0.2 ha). In Ukraine, atmospheric emissions of carbon dioxide per capita are three times the world average, while the average atmospheric pollution in the country is 6.5 times higher than in the USA (Didukh, 1997; Holubets, 2003; Isachenko, 1991; Morhun and Hryhoriuk, 1997). The average life expectancy in Ukraine is 10 to 12 years less than in most European countries, while the average wages are 100 times lower than the average European pay. Furthermore, Ukraine has the greatest area of man-made lakes, erosive reclaimed lands and plant fragmentation (Herenchuk, 1981; Hryhoriuk et al., 1999; Sheliah-Sosonko, 1987; Mezherin et al., 1991; Morhun and Lohvynenko, 1995). This situation has been aggravated by the illegitimate transportation of detrimental and toxic waste into Ukraine and by the concealment of information on ecological catastrophes.

The Carpathians, like the Alps, are one of the mightiest and most interesting mountain systems in Eurasia from the geological, geomorphological, landscape and biogeographical viewpoints. The gigantic Carpathian arc starts in Southern Romania and stretches across Ukraine, Slovakia, Poland, the Czech Republic, Hungary and Austria, thus touching the whole of East and Central Europe. The total area of the mountain system is 204,700 km^2, with a length of 1,500 km, and a width varying from 100 to 350 km. The highest peak is Mt. Gerlach (2,655 m above sea level) in the Slovakian Tatras. On the basis of their geographical location and biogeographical features, the Western, Eastern and Southern Carpathians can be distinguished. The Western Carpathians stretch across Slovakia, Poland, the Czech Republic, Hungary (Pre-Mátra Phytogeographical Region) and Austria. The Eastern Carpathians extend from the Oslava and Laborec Valleys in Slovakia through Ukraine to the Bucegi Mts. and Predeal Overpass (1,033 m above sea level) in North Romania. The Southern Carpathians (with the phytogeographical regions of Apuseni and the Transylvanian Mts.) are fully located within Romania. Over a half (55%) of the total area of the Carpathian Mts. is located in Romania, 17% in Slovakia, 11% in Ukraine, 10% in Poland, 4% in Hungary, 3% in the Czech Republic and less than 1% in Austria.

Owing to their geographical position, vast area and rich natural heritage, the Carpathians are vitally important for the preservation of biological, phytocoenotic and landscape diversity and for the maintenance of the ecological balance in the central part of Europe. Together with the Alps, this mountain system is an important ecological corridor between West, Central and East Europe, which promotes the migration of biological species and their expansion into the flat countries. The natural forest landscapes of the Carpathians serve as a refuge for numerous species of large mammals whose West European populations have either become extinct or are endangered.

The mountain system is located in a humid climatic zone with an annual precipitation of 1,600–1,800 mm. The sources of major rivers such as the Visla, Dniester, Prut and Aluta, and of tributaries of the Danube such as the Tisza and Vág, are found in the Carpathians. The Carpathian Mts. represent an important European watershed between the Baltic and the Black Sea basins. Due to their significant role in water and soil protection and in climate regulation, the mountain forests are of great importance for the maintenance of the standard hydrological regime and the prevention of dangerous floods.

When using the natural resources of the Carpathians, it is worth bearing in mind that mountain ecosystems are extremely sensitive to human impact. Hillslope processes intensify the groundwater run-off and develop gully and surface erosion with a permanent threat of avalanches. The steeper the slopes, the more evident the hillslope processes are. Due to the constant activity of these processes, the homeostatic ability of mountain ecosystems (i.e. their ability to maintain an ecological equilibrium) is far lower than that of the plains. Therefore, repairing the damage to mountain ecosystems is more complicated and protracted than in flat countries (Herenchuk, 1981; Grodzinski, 1993; Zastavny, 1994; Sheliah-Sosonko, 1987; Komendar, 1966). However, when emphasising the problems of the Carpathians, the ecological problems of the plains and rivers adjacent to the mountains cannot be ignored.

Social and ecological aspects of the mountain system

Archaeological data prove that human settlements were founded in the warmer parts of the Carpathian valleys and in their foothills about 5,000 years ago. The ethnic groups living there 2,000 years ago were mentioned in Roman manuscripts. The severe nature of the mountains had a considerable influence on the way of life of the locals, who had to work hard to expand the available arable land and pastures. Currently, there are 16 to 18 million people living in the Carpathian region. They are ecologically and economically dependent upon renewable and non-renewable resources. This is a multi-ethnical region inhabited by Romanians, Slovaks, Ukrainians, Poles, Hungarians, Czechs, Germans, Romany, etc., who are distinguished by a unique way of life, with special traditions, customs and culture. It is the task of sociologists to preserve this rich ethnical and cultural heritage, notwithstanding the contemporary global urbanization and the spread of technology.

Since the end of the 20th century, political and economic changes in Central European countries have caused certain changes in the lifestyle of these people. Owing to the market economy, the development of civil society, political and religious freedom, and the desire for integration with West European countries, their mentality has changed. Low living standards, however, and the unprofitability of farming have remained an important obstacle to the economic development of the region. Thus, taking into consideration the political and economic changes that have taken place in the Carpathian region, and in the mentality and democratic thinking of the mountain dwellers, a new socio-economic development strategy should be compiled for different branches of the national economies. In this respect, the experience gained by dwellers in the Swiss, Austrian and French Alps, who live in a similar environment involving mountain crop farming and stock-raising, should be given special attention. Economic, ecological and cultural contacts between the inhabitants of these regions couled lead to the rich economic experience acquired in the Alps being made available to the economies of the Carpathian region. If promising branches of the economy are to be developed under new socio-economic and political conditions, certain investment aid from the EU will be required.

Natural heritage of the mountain system and how to preserve it

Due to their diverse geological and geomorphological structure and favourable soil and climatic conditions, the Carpathians are characterised by significant biological diversity. The regional flora consists of over 3,980 species and subspecies of vascular plants, or 31.2% of the European floristic wealth, known to include 12,500 taxons. The Carpathian flora includes 502 endemic species and subspecies (12.6% of its species composition), the protection of which is a priority for ecologists, for if they should be lost, European biodiversity would be much poorer.

Owing to the substantial areas of wild and underdeveloped landscapes, the Carpathians are distinguished by a rich species composition of the vertebrates and invertebrates. The Carpathian region represents the biggest European refuge for populations of such large mammals as the brown bear *(Ursus arctos),* the wolf *(Canis lupus),* the lynx *(Lynx lynx),* the chamois *(Rupicarpa rupicarpa),* the steppe marmot *(Murmota marmota)*, etc., which have become extinct in Western Europe. Recently, a population of bison (currently consisting of over 200 animals) has been successfully reintroduced in the Polish (Beszczady National Park) and Ukrainian Carpathians (Lviv and Ivano-Frankivsk regions).

In order to preserve biological and phytocoenotic diversity and the unique natural landscapes suitable for recreational purposes, a wide network of reserves of different types has been organized in the Carpathian region. Nineteen national nature parks, five biosphere reserves and over 400 smaller forest reserves have been set up. By 1996, from 10 to 14% of the area of the Carpathian region had been protected. Descriptions of the national parks and other reserves were given by Stoiko et al. (1991).

Taking into consideration that the Carpathian Mts. spread across the territories of seven countries, it is important from the ecological viewpoint to protect the natural ecosystems in trans-frontier localities. A bilateral biosphere reserve, *The High Tatras,* was organized in the Slovak and Polish Tatras; another biosphere reserve, Aggtelek, was set up in the Slovak–Hungarian frontier area. In 1999, *The East Carpathians* (208,000 hectares) trilateral biosphere reserve was founded in the Polish–Slovak–Ukrainian frontier area. International conferences devoted to the Carpathian region proposed founding a Ukrainian–Hungarian natural landscape park in the river Tisza basin, and a bilateral Ukrainian–Romanian reserve, *The Maramorosh Mts.,* in the frontier area of the respective countries. The trans-border reserves will be helpful not only for the mutual solution of ecological tasks, but also for the mutual cultural enrichment of the local population. Due to the powerful mining industries located in the Romanian frontier areas, and the threat that sedimentation reservoirs will be polluted by highly toxic industrial refuse, it has become extremely important not only to preserve the existing ecosystem, but to ensure the ecological safety of the inhabitants of the Ukrainian and Hungarian territories located within the Tisza basin.

Remote spots in the Carpathian Mts. have preserved approx. 60,000 hectares of beech *(Fagetum sylvaticae),* fir–beech *(Abieto–Fagetum),* beech–fir–spruce *(Fageto–Abieto–Piceetum)* and spruce *(Piceetum abietis)* forest ecosystems with exceptional significance for natural science. Apart from their inherent value, they constitute a natural model for the reconstruction of secondary, biologically instable phytocoenoses and efficient nature-based forest management.

In order to preserve biological diversity, a joint effort should be made to develop an All-Carpathian Red Book of rare species of flora and fauna. An inventory should be made of genuine forest ecosystems, which should be preserved. Further bilateral reserves should be set up in trans-border areas to try and solve the most crucial environmental tasks.

Taking into account the biogeographical and landscape uniqueness of the Carpathian mountain system and its significance for the maintenance of the ecological balance in Central Europe, the World Wildlife Fund (WWF) has included it in the world's 200 ecologically most important regions (Program Global-200). In October 2002 in Liechtenstein, international experts considered the issue of the Framework Convention on Protection and Stable Development of the Carpathian Mts. The Convention was approved in Kiev, Ukraine in May 2003. It substantiated the ecological basis for the preservation and continuous use of biological diversity, the development of agriculture, forestry and water industry, the improvement of tourism, the conservation of the cultural heritage, and other tasks of importance for the stable development of the region (Anonymous, 1998; Didukh, 2000; Didukh et al., 2000; 2004; Morhun and Lohvynenko, 1995).

Problems of forest management and forest protection

Europe has lost 56% of its former forest areas. Only 2% of its forests are protected, and it is in the Carpathian region that the largest European beech and beech-and-spruce forest ecosystems have remained, as well as the biggest European genuine forests (Morhun and Lohvynenko, 1995; Nikolaichuk, 2002).

For thousands of years, the Carpathian forests have been producing oxygen and contributing to the increase in air humidity needed to form freshwater reserves for the Zakarpatska region of Ukraine in particular and for the whole Tisza-Danubian basin of Hungary and Central and East Slovakia. In recent centuries, the Carpathian forests have undergone undesirable quantitative and qualitative changes, which considerably affected the ecological stability of the environment. The areas of oak, beech and spruce forests have steeply declined, with a simultaneous increase in fir afforestation (predominantly monocultures), whose water-regulation properties are 7 to 17 times lower than those of beech forests. Alluvial forests able to retain and accumulate over 50% of the water in the plains are continuing to disappear. Due to the lengthy pasturing period in the Carpathian Mts., the upper timber-line, whose water-protection function is far higher than that of the phytocoenoses located on lower slopes, has significantly receded, now being 200–300 m lower (Morhun and Lohvynenko, 1995).

To correct this situation, an urgent reconstruction of the forest management system will be required in order to achieve the intensification of forestry, the introduction of environment-friendly, water-collecting principles of management in mountain areas and the launching of measures intended to improve the age, spatial, specific and ecotypical structures of the forests at all stages of forest use and reproduction. A system that considers zonal, natural and economic conditions, and the probable changes in the forest environment due to human activity and urbanization, will contribute to the fuller and more efficient use of forest lands, improvements in forest productivity and composition, an increase in the level of forest exploitation, and the simultaneous preservation and reinforcement of the water-protection function and other useful properties of forests (Isachenko, 1991).

Heavy metals

The discharge of cyanide into the Tisza Basin due to accidents at mines located in Baia Borşa, Romania, has inflicted huge ecological damage. The contaminants spilled into the River Ţisla, a tributary of the Vişeu, through gaps in the dam, and spread into the Vişeu and Tisza. Following the breach in 2001, the pollution greatly exceeded the maximum safe concentrations, by 200 times for Cu, 10 times for Zn, 14 times for Pb, 60 times for Mg and 620 times for Fe. Most seriously, the heavy metals contaminated the water and soils used for vegetable gardening in the Tisza Basin.

When entering the bodies of warm-blooded animals, heavy metals and their compounds cause a broad spectrum of disorders, characterized by a high frequency of allergization, intoxication syndromes, immune deficiency, microbiocoenosis changes, the activation of opportunistic pathogenic microflora, mycoses of the skin, mucus and viscera, etc. New monitoring techniques should be introduced to detect pollution levels and to gauge their effects on the ecosystems and population of the Tisza Basin under the given climatic conditions.

The numerous motorways that run through the Transcarpathian region are a further source of pollution. The problem of the toxic effect of heavy metals is especially acute in the lower course of the Tisza, where families have been consuming polluted agricultural products for years. The lead content in wheat and barley grains was observed to be 5–8 times the permitted limit, while this figure was 4–7 times for potatoes and 5–10 times for other agricultural products.

It is thus extremely urgent to investigate the accumulation of chemical elements by plants in the polluted areas, in order to determine the negative impact of the food chain on the population. Recommendations are being elaborated on a decrease in the environmental pollution level and on measures to ensure the manufacture of ecologically pure agricultural products.

Water supply, water pollution and catastrophic inundations

In Ukraine, which ranks very low in the world in terms of per capita surface water supplies, over 2.5 billion cubic metres of polluted water are discharged annually into reservoirs and watercourses, nearly half of which comes from urban community facilities and another 28% from the iron industry. On average, the annual discharge into Ukrainian reservoirs and watercourses includes 5 million t salts, 5,000 t oil products, 7,800 t phosphorus, 130,000 t organic pollutants and 1,400 t synthetic surface-active agents.

In the Zakarpatska region, it is mainly urban communities that have a central water supply and sewerage, but approx. 30% of the sewage system is in very poor condition. Nearly half the treatment facilities need reconstruction to increase their discharge capacity and to introduce more advanced wastewater treatment technologies.

The Ukrainian part of the Transcarpathian region is prone to flooding. The steep mountain slopes cause floods to develop rapidly: within 3–4 hours, water levels may rise by up to 1.5–2.5 m. When the flood waters reach the plains, vast land areas are inundated, causing great losses to the economy of the Zakarpatska region.

There are many reasons for the occurrence of floods in the Carpathian Mts. According to a group of experts, the floods in November 1998 and March 2001 were caused by at least five natural and 13 anthropogenic factors, including the climate, the land surface geology, relief and orography, the vegetation cover, the siting, organization and population density of villages, and human activities in the river basins. However, every flood also had its own specific features.

This problem became especially acute during the destructive flood in March 2001, which destroyed dozens of kilometres of riverside greenery, formed hundreds of sources of silt and gravel, changed the beds of the rivers and streams, and annihilated hundreds of hectares of homesteads and arable lands.

As the hydroresources of the Zakarpatska region are an integral part of the European water basin, and as the negative effect of regional phenomena will not stop at the state border, but will make themselves felt in the whole continent, there is every justification for the financing of research into these resources to be shared by EU and Ukrainian institutions.

Atmospheric pollution

The main cause of atmospheric pollution is the obsolescence of technological equipment, the inefficiency of dust-and-gas-trapping units, and the lack of preventive repair works on compressor plants, causing an increase in emissions.

Motor transport has long been the main atmospheric pollutant in the Zakarpatska region. Exhaust fumes make up 70.1% of the total volume of atmospheric emissions. The situation is likely to become worse, as the number of private cars is growing very quickly, so strict ecological control of the contents of hazardous substances in exhaust gases will be required.

Use of the mineral wealth

The Zakarpatska region contains a large share of the mineral wealth and other raw materials in Ukraine due to its specific geological history and geological composition.

In recent years, there has been a tendency to re-open old mineral resource plants and to establish new ones in the area. The existing mining plants in the region are located mainly in the lowlands and foothills, with only approx. 10% within the Carpathian mountain range. Notwithstanding the fairly insignificant bedding depth of the mineral resources, their extraction may lead to irreversible changes in the areas where mining industries are located. Significant volumes of the minerals extracted from the rocks, together with their further processing, have often had a negative effect on the environment of the region.

The allocation of agricultural (arable) lands for the needs of the mining industry is an important ecological and economic factor which is often ignored. The loss of such lands is usually irreversible.

Use of genetically modified plants

Due to globalization processes, problems of biological safety are gradually coming to the fore. The use of genetically modified products (GMO) is deemed to be a constituent part of this problem.

GMO use should be regulated to minimise the risk to human health and the environment. The risk assessment approach must include the following: identification of the hazardous properties of GMOs; assessment of the consequences for human health and the environment; risk management procedures to reduce the probability of harm; assessment of the negative consequences of GMOs entering the environment; and methods for eliminating them (Nikolaichuk and Sharga, 2003; Sheliah-Sosonko, 1987).

Waste management

Warehousing and burying are the most widespread and cheapest means of waste management. The current state of the disposal tips (dumps) in the Zakarpatska region is far from satisfactory. As a result, the garbage is spread by the wind and by birds, contributing to environmental pollution. Unauthorized disposal tips, especially those located near small settlements, constitute another big problem, as the garbage is often dumped near waterways, and even the most insignificant floods wash it into the rivers. A large amount of household and, sometimes, industrial waste enters the water and is carried great distances. Waste disposal by burning is mostly conducted by the local population using primitive means, and this process has never been controllable. The huge potential for the mechanical, chemical and biological treatment of hazardous substances has never been applied in practice (Nikolaichuk, 1998; 2002; 2004).

In Hungary, there has recently been a qualitative change in the approach to waste management. Numerous small-size dumps have been substituted by large regional disposal tips that comply with contemporary ecological standards. However, the banning of waste storage in one country tends to lead to its being transported to another country with less strict ecological controls, under cover of business deals. This is how the "*premix*" imported as raw material for the rubber industry entered Ukraine. Between 1999 and 2005, approx. 1,500 tons of this hazardous waste were imported into the Zakarpatska region, causing a huge ecological, social and financial problem. According to the results of analyses, the imported substance is a highly toxic product, as it contains such inorganic substances as lead, copper, chromium and nickel, and correspondingly needs special recycling techniques.

Impact of environmental factors upon human health

The current unfavourable demographic situation and unsatisfactory socio-economic situation in the country have contributed to the deterioration of public health. There has been a rise in sickness rates in most regions, primarily due to circulatory diseases, endocrinal problems (especially involving the thyroid), haematological ailments, and diseases of the respiratory system and alimentary organs.

Tourism and recreation

Tourism in the Carpathian Mts. is both a highly promising factor in regional development and a significant ecological problem, especially in areas known as "hotspots" (Didukh, 2000).

Undoubtedly, a stable tourist industry would have huge potential to combine natural conservation with rural development. Tourists will be encouraged to visit the Carpathians, but ecological damage must be prevented by providing satisfactory facilities and by penalising violations. The income generated must be used for the improvement of recreation facilities.

Preservation of ethnic and biological diversity

Mountain systems, as the source of biological species and the cradle of nations and cultures, occupy a special place in the development of civilization. Mountain terrains have a decisive influence on the climate, water regime and the whole ecology of the surrounding plains. In addition, ethnic and biological diversity tend to be better preserved in mountain systems than in the lowlands.

Conclusions

It is in the Ukrainian Carpathians that the biggest European temperate forests have been preserved. These forests have not yet been exposed to the detrimental impact of civilization, mainly due to the broad, well-organized system of state reserves and wildlife preserves, combined with the harmony between nature and the local population, with its deep cultural traditions. This natural reserve is of great importance for the preservation of the regional wildlife, but the assimilation of the adjacent areas and the growing disregard of the local population may result in the gradual decline of these natural islands.

This is of particular importance in post-Soviet Ukraine, when society is neglecting rich cultural traditions and ignoring the annihilation of unique natural areas, which may disappear for ever due to being parcelled off as agricultural land. To lessen the danger and preserve the integrity of this ecological system in the long term, a complex environmental programme will be needed to stop the territory of the reserve from being fragmented and to retain the agricultural use of the adjacent lands. Such measures could provide for the following:

1) improved possibilities for the development of other economically valuable territories that do not infringe on parts of the reserve;

2) a gradual transition from protected territories to agricultural lands;

3) the positive effect of suitable economic activities on natural systems;

4) the preservation of the cultural traditions of the local population.

It will only be possible to protect and preserve the Carpathian countryside for our own and later generations if there is close cooperation with the whole European region, based upon the principle of minimum interference with nature and involving an increase in the number of trans-border reserves.

An international ecological network should be set up for the identification of key regions, ecological corridors, renewable and buffer areas, and for the selection of well-preserved ecosystems and specific habitats of regional, national and international significance. The whole strategy of forest management should be critically reviewed to achieve the gradual transformation of forest "agrosystems" into natural ones. A uniform state and international system should be devised to control the use of GMOs. Scientific solutions must be found to the problem of environmental pollution from transport and the energy sector. Farming must be reoriented to provide for stable economic development with a minimum of negative environmental effects. Finally, uniform environmental laws will be required for all European nations, including sanctions for those who fail to comply with them.

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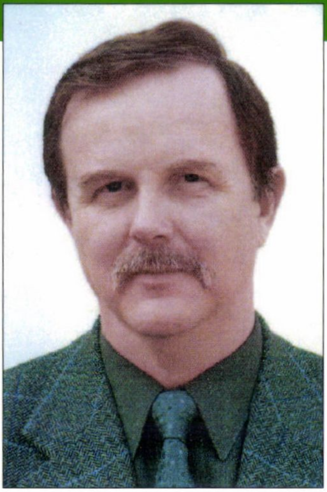

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EFFECT OF SOWING DATE AND N FERTILISATION ON THE YIELD AND YIELD STABILITY OF MAIZE (*Zea mays* L.) HYBRIDS IN A LONG-TERM EXPERIMENT

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The effect of sowing date, N fertilisation and genotype on the grain yield and yield stability of maize was studied between 1991 and 2006 in a long-term N fertilisation experiment set up on chernozem soil in Martonvásár, Hungary. The N treatments (0, 60, 120, 180 and 240 kg ha^{-1}) represented the main plot of the three-factor, split-split-plot experiment, with the sowing date (early, optimum, late, very late) in the sub-plots and hybrids from different maturity groups in the sub-sub-plots. The highest yields were obtained for the early and optimum sowing dates (8.712 and 8.706 t ha^{-1}). Compared with the optimum sowing date, a delay of ten or twenty days led to yield losses of 5% and 12.5%, respectively. In the late and very late sowings and in years with unfavourable weather conditions, yield increments were only observed up to an N rate of 60 kg ha^{-1}, while in the early and optimum sowings and in favourable years yield increments were significant up to 120 kg ha^{-1} N. Yield stability was smallest in the early and very late sowings, in the control and for high N rates, and in the early and late maturity hybrids. It can be concluded that high yields and yield stability are not mutually exclusive.

Key words: maize, sowing date, long-term experiment, interaction, stability analysis, AMMI model

Introduction

Changes in the environment that are associated with different sowing dates can be expected to alter maize growth and development in temperate regions. Radiation and thermal conditions become progressively unfavourable for plant growth during the reproductive period when sowing date is delayed. Sowing date determines the environmental conditions to which the maize crop is exposed during grain filling (Cirilo and Andrade, 1994, 1996). Grain yield in maize is mostly dependent on variations in the number of kernels harvested (Tollenaar, 1977; Westgate and Boote, 2000). However, growth conditions during grain filling could also affect grain yield by affecting dry matter allocation to the kernels (Tollenaar and Daynard, 1978; Otegui et al., 1995).

For each successful maize genotype there exists an optimum date of sowing, and sowing before or after that optimum date results in yield reduction. The scientific literature suggests that a trend towards earlier maize planting has taken place over the past few decades, which can be largely attributed to the continued development of genotypes that are tolerant of suboptimal temperatures, improvements in planting equipment, and the adoption of time-saving management practices such as conservation tillage. Maize sowing now begins approximately 2 weeks earlier on average, relative to the early 1980s (Kucharik, 2006). Because early sowing increases the time period during which plants can absorb solar radiation, perform photosynthesis and accumulate biomass, higher yields are generally achieved where the growing season is longest and soil moisture is not limiting. Early sowing allows later maturing hybrids with higher yield potential to be used, as long as they are tolerant of low (non-freezing) temperatures after sowing. Early sowing also increases the likelihood that physiological maturity will occur before killing frosts occur in autumn and contributes to lower grain moisture, leading to reduced drying time and energy costs, now a major concern for growers due to rising fuel prices (Lauer et al., 1999; Szundy et al., 2005).

If excessive rainfall occurs before or during the planting season, sowing may be delayed beyond the optimum time frame (mid-April to early May). Occasionally, fields planted during the optimum time frame require replanting at later dates after weather stress or pests cause excessive plant mortality. Because delayed planting and replanting shorten the effective growing season, it may be necessary to switch to early-maturing hybrids to ensure that physiological grain maturity occurs before a killing frost (Nielsen et al., 2002).

Maize must be properly managed (selection of appropriate hybrids, planting dates and plant populations) to tolerate the low precipitation and high temperatures that limit yields in dry regions (Norwood, 2001), particularly if they occur during the flowering period. Most growers agree that dryland maize should be planted early so that it can be pollinated before high midsummer temperatures and drought stress occur. Planting maize before or after the optimum date was found to result in reduced leaf area index, leaf area duration, total dry matter production and grain yield. More recent research (Nafziger, 1994) has shown an accelerating decline in yield as the planting date is advanced or delayed from the optimum.

Stability analysis is often used to interpret the significant treatment × environment interactions observed in the models used for analysis of variance in long-term trials and experimental series. The measurement of yield stability over time involves at least three components: (i) the relationship of yield with local environment, (ii) the average yield level and (iii) the variability of the yield (Mead et al., 1986). The mean (fixed, i.e. systematic effect) and the variance (random element) are the two main parameters describing the response pattern of a cropping system (Piepho, 1998). The larger this random component, the smaller the stability of a system. Different approaches to stability analysis differ in how the random term is further partitioned.

The aim of the research was (i) to determine the effect of sowing date, hybrid and N fertilisation on the grain yield of maize and on some agronomical and ecophysiological characteristics of maize plants and (ii) to analyse the stability of maize yields using the univariate (variance and regression parameters) and multivariate (AMMI) methods of stability analysis.

Materials and methods

Experimental design

The effect of sowing date, N fertilisation and genotype on the grain yield of maize was studied between 1991 and 2006 in a long-term N fertilisation experiment set up in 1980 in the experimental nursery of the research institute in Martonvásár, Hungary (47°21' N, 18°49' E). The soil of the experimental area, a humous clay of the chernozem type with forest residues, was slightly alkaline in the ploughed layer, with a humus content of 3.3–3.6% and good supplies of phosphorus and potassium. In the three-factor, split-split-plot experiment the N fertiliser treatments represented the main plots, with the sowing date in the sub-plots and the maize hybrid in the sub-sub-plots. The N treatments were as follows: 0, 60, 120, 180 and 240 kg ha^{-1} (designated as N_0, N_{60}, N_{120}, N_{180}, N_{240}), while all the treatments received 120 kg ha^{-1} each of P and K. Sowing took place at four dates: 10 days prior to the optimum date (early, S_1), at the optimum date (around April 24, optimum, S_2), ten days after the optimum date (late, S_3) and 20 days after the optimum (very late, S_4). The four sowing dates (at 10-day intervals) applied in the experiment all represent realistic dates when sowing is carried out in practice. The actual sowing dates in the experimental years are presented in Table 1. Each year four commercial hybrids were examined, chosen to represent different maturity groups: FAO 200–299: *Mara TC* (FAO 290) until 1998 and *Mv TC 272* (FAO 280) from 1999, designated as H_1; FAO 300–399: *Norma SC* (FAO 370), grown throughout the experiment (H_2); *Furio SC* (FAO 390) until 1994 and *Mv 355 SC* (FAO 390) from 1995 (H_3); FAO 400–499: *DK 524 SC* (FAO 460) until 1994 and *Maraton SC* (FAO 450) from 1995 (H_4). The main plot measured 30 m × 6 m and the sub-plot 7.5 m × 6 m, while the hybrids representing the sub-sub-plots were each grown in two rows, separated by buffer rows.

Traditional agronomic practices including deep ploughing in autumn were carried out on the experimental area. The herbicides used for weed control each year were 2.0 kg ha^{-1} atrazine [6-chloro-N-ethyl-N'-(1-methylethyl)-1,3,5-triazine-2,4-diamine] + 2.5 l ha^{-1} acetochlor [2-chloro-N-(ethoxymethyl)-N-(2-ethyl-6-methyl-phenyl)acetamide] applied pre-emergence. For early post-emergence treatments nicosulfuron [2-[[[[(4,6-dimethoxy-2-pyrimidinyl) amino] carbonyl] amino] sulfonyl]-N,N-dimethyl-3-pyridinecarboxamide] was applied at a rate of 0.7 l ha^{-1}. The seed were sown with a Wintersteiger plot drill at a density of 70,000 plants ha^{-1}. Continuous maize had been grown since the start of the experiment. The crop was harvested with a Bourgoin plot combine harvester.

Table 1

Calendar dates for the early, optimum, late and very late sowings in different years

Year	Sowing dates				Year	Sowing dates			
	Early	Optimum	Late	Very late		Early	Optimum	Late	Very late
1991	12 Apr.	22 Apr.	2 May	12 May	1999	9 Apr.	20 Apr.	30 Apr.	10 May
1992	17 Apr.	27 Apr.	7 May	18 May	2000	17 Apr.	27 Apr.	9 May	19 May
1993	21 Apr.	1 May	11 May	21 May	2001	13 Apr.	23 Apr.	3 May	14 May
1994	16 Apr.	26 Apr.	7 May	16 May	2002	16 Apr.	26 Apr.	8 May	17 May
1995	12 Apr.	22 Apr.	2 May	13 May	2003	14 Apr.	25 Apr.	6 May	16 May
1996	15 Apr.	25 Apr.	6 May	16 May	2004	20 Apr.	29 Apr.	10 May	20 May
1997	16 Apr.	26 Apr.	5 May	16 May	2005	16 Apr.	27 Apr.	6 May	17 May
1998	14 Apr.	24 Apr.	6 May	15 May	2006	13 Apr.	24 Apr.	4 May	15 May

Agronomical and ecophysiological measurements

Each year the date of silk emergence was defined as the date by which 50% or more of the plants exhibited silks. The N status of the plants was measured between 2001 and 2006 using a SPAD 502 (Minolta) chlorophyll meter. Measurements were made after silking on the leaf beside the ear on 30 plants per plot. The measurement of photosynthesis was carried out using a portable LCA-4 (ADC, England) photosynthesis system during silking between 2002 and 2005. Measurements were made on three plants per plot, on the leaf next to the ear, between 10 a.m. and 2 p.m., in bright sunshine whenever possible. The radiation intensity during the photosynthesis measurements had the following mean values (μmol m^{-2} s^{-1}): 2002: 1337, 2003: 1570, 2004: 1598, 2005: 1659. Between 2001 and 2006 the yield components (kernel number, kernel mass) were determined for five sample ears per plot. The grain moisture content was recorded at harvest each year and the yield data were converted to 15.5% grain moisture content.

Statistical analysis

The experimental data were evaluated using the GenStat for Windows (11th edn.) program. In the first step the yield data were evaluated each year using the General Analysis of Variance Split-Split-Plot Design menu. Before beginning a combined analysis of variance over years the homogeneity of the error variance in different years was examined. A quick test of the homogeneity of variance is provided by the ratio of the largest to the smallest s^2 in the set. The ratios of variances were the following: for main-plot error (a): 4.95, for sub-plot error (b): 8.18, for sub-sub-plot error (c): 3.33. According to Williams et al. (2002) a 10-fold range of residual mean squares can be tolerated when forming a pooled residual over sites or seasons. As the variances were not heterogeneous, the combined analysis of variance over years was carried out to determine the main effects and interactions of the treatments. In the next step the 16 years were divided into favourable (9) and unfavourable (7) years on the basis of rainfall quantity and distribution and ANOVA was carried out for each group. Contrasts (polynomial, group comparisons) were incorporated into the ANOVA model. The structure of the combined ANOVA was based on Gomez and Gomez (1984).

The stability analysis of the experimental treatments was carried out using univariate (variance and regression parameters) and multivariate (AMMI) methods. The year × treatment interaction was examined using AMMI analysis (Crossa, 1990; Kang and Gauch, 1996). The first part of the AMMI analysis involves partitioning the total variance into three orthogonal sources: genotype (G), environment (E) and the G × E interaction, while the second step uses principal component analysis (PCA) to divide the G × E interaction into orthogonal principal component variables (PCA axes). The position of the treatments (G) and years (E) on the X axis is indicative of the mean yield response, while the PCA1 values demonstrate the contribution of the treatment to the G × E interaction. The greater the PCA1 value, the greater the contribution of the treatment to the interaction, i.e. the smaller the yield stability.

Univariate stability measures use either variance or regression methods. The following variance parameters were calculated using the STABLE model of Kang and Magari (1995): the coefficient of variation (CV %), the ecovalence index (W^2) (Wricke, 1962), the stability variance (σ^2) (Shukla, 1972) and the yield stability (YS) (Kang, 1993). The ecovalence index (W^2) and the stability variance (σ^2) measure the contribution of the treatment to the treatment × environment interaction (Callaway and Francis, 1993), while the yield stability (YS) statistic combines yield and stability of performance into a single selection criterion.

In the regression method of stability analysis the regression between the experimental treatment and the environmental index is calculated. The environmental index is the mean of each treatment in a given environment (year), expressing the productivity of the given location. Linear regression analysis was carried out according to the methods of Finlay and Wilkinson (1963) and Raun et al. (1993). It was suggested by Finlay and Wilkinson (1963) that a regression coefficient of $b<1.0$ indicated better adaptation to unfavourable environments, while a value of $b>1.0$ was characteristic of genotypes that can best be grown in favourable locations.

Results

Climatic conditions

The monthly rainfall (mm) and mean temperature (°C) figures for the growing period, between April and September, are given for the years between 1991 and 2006, together with the 30-year mean, in Table 2. Averaged over 16 years, the rainfall sum during these months was 324 mm, which was slightly higher than the 30-year mean. The lowest rainfall sums were recorded in 2000 and 2003 (186 and 178 mm) and the highest in 1998 and 2005 (502 and 527 mm). Averaged over the 16 years, the mean temperature between April and September (17.5°C) was similar to the 30-year mean, ranging from 15.6 and 16.0°C in the coolest years (1996, 1995) to 19.8 and 18.5°C in the hottest years (2003, 2005). The number of days with temperatures of 30°C or above was lowest (between 7 and 16) in 1995, 1996 and 1999 and highest (47 and 70) in 1992 and 2003. Based on the quantity and distribution of rainfall the 16 years can be divided into unfavourable (1993, 1994, 1997, 2000, 2001, 2002, 2003) and favourable (1991, 1992, 1995, 1996, 1998, 1999, 2004, 2005, 2006) years, and the effect of the treatments on the grain yield was also evaluated from this point of view. The rainfall quantity averaged 247 mm in the unfavourable years and 384 mm in the favourable years, while the mean temperature between April and September was 17.9°C in unfavourable years and 17.2°C in favourable years. The number of very hot days averaged 45 in the unfavourable years and 26 in the favourable years.

Table 2

Monthly rainfall (mm) and average air temperature (°C) during the maize growing period in Martonvásár, Hungary, 1991–2006

Month	Avg.+	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006
									Rainfall								
April	43	26	19	19	93	54	39	30	97	76	63	24	61	15	64	97	34
May	56	52	14	30	54	35	93	62	92	35	20	15	34	17	49	70	62
June	73	41	210	16	19	82	47	38	64	163	7	64	29	23	98	49	56
July	53	86	43	76	32	25	28	69	70	123	57	67	80	68	45	76	42
Aug.	46	72	0	30	50	100	51	11	43	63	6	18	80	37	28	186	109
Sept.	41	8	29	57	41	59	112	15	136	17	33	108	42	18	14	49	21
Total	312	285	315	228	289	355	370	225	502	477	186	296	326	178	298	527	324
									Temperature								
April	11.3	8.8	11.8	10.6	10.8	9.8	10.3	8.2	11.9	12.1	13.7	10.5	11.4	13.4	11.7	11.1	14.0
May	16.4	12.5	16.7	17.8	16.1	14.6	16.1	17.2	15.1	15.8	17.2	18.5	18.7	19.7	14.9	16.3	15.2
June	19.8	18.2	20.6	18.9	19.3	17.3	17.9	18.7	20.1	19.2	20.5	18.2	20.1	22.7	18.6	18.7	20.0
July	21.5	21.9	21.3	19.3	22.6	21.3	17.5	19.1	21.0	21.7	20.1	21.8	22.0	22.1	20.9	21.4	24.0
Aug.	20.7	19.7	23.9	20.5	20.2	19.4	19.0	20.1	21.0	19.9	22.4	23.1	21.3	23.6	21.0	19.8	19.0
Sept.	16.6	16.3	16.2	15.4	16.9	13.7	12.6	15.4	15.5	18.9	16.1	14.8	16.5	17.5	16.1	17.4	18.5
Mean	17.7	16.2	18.4	17.1	17.7	16.0	15.6	16.5	17.4	17.9	18.3	17.8	18.3	19.8	17.2	17.5	18.5

+30-year mean

Effects of N fertilisation, sowing date and hybrid on the maize grain yield

Table 3 illustrates the significance of the F values on the main effects and interactions, between 1991 and 2006, based on the results of annual ANOVA. The effect of N fertilisation on the grain yield was significant every year with the exception of 1991, and was shown by the MS values to exceed that of the sowing date in all but two years. The effects of sowing date and hybrid were significant every year. Among the first-order interactions, the N fertiliser × sowing date interaction was significant in four years and the N fertiliser × hybrid interaction in seven years. With the exception of four years, the sowing date × hybrid interaction was significant every year, i.e. the hybrids investigated gave different responses to changes in the sowing date in 12 of the years. The second-order interaction (N fertiliser × sowing date × hybrid) was significant in five years, but the MS values showed it to be of slight importance.

When examining the annual effect of N fertilisation the lowest yields were obtained in the control treatment without N fertilisation. N rates of 60 and 120 kg ha^{-1} led to significant increases in yield, which then stagnated at the 180 kg ha^{-1} rate and significantly declined at 240 kg ha^{-1} N. The only exception was 1996, when the significantly highest yield was achieved at a rate of 240 kg ha^{-1} N. In general, the highest maize grain yields were achieved in the early and optimum sowing dates, with significant reductions in the late and very late sowings. The only exceptions were 1999 and 2001, when the greatest yields were recorded for the late sowing date. Hybrids with longer vegetation periods tended to have significantly higher grain yields than those with shorter vegetation periods.

Table 3

Effect of N fertilisation, sowing date and hybrid on the significance of F-values in the three-factorial split-split-plot experiment (1991–2006)

Treatments	1991	1992	1993	1994	1995	1996	1997	1998
N fertilisation (N)	NS	***	*	**	***	***	***	***
Sowing date (S)	*	***	***	**	***	***	***	***
Maize hybrid (H)	***	***	***	***	***	***	***	***
N × S	NS	NS	NS	NS	NS	NS	NS	**
N × H	NS	NS	NS	NS	NS	NS	*	*
S × H	**	***	NS	NS	***	***	***	NS
N × S × H	NS	**	NS	NS	NS	**	NS	NS
Treatments	1999	2000	2001	2002	2003	2004	2005	2006
N fertilisation (N)	***	***	***	***	***	***	***	***
Sowing date (S)	**	***	***	**	***	***	***	***
Maize hybrid (H)	***	***	***	***	***	NS	***	***
N × S	NS	*	NS	NS	NS	***	***	NS
N × H	***	NS	***	NS	***	NS	***	*
S × H	**	***	**	**	***	***	***	NS
N × S × H	NS	*	***	NS	NS	NS	***	NS

NS = not significant at $P \leq 0.05$, * Significant at $P \leq 0.05$, ** Significant at $P \leq 0.01$, *** Significant at $P \leq 0.001$

Combined analysis of variance on the three-factor, split-split-plot experiment was carried out on all 16 years, and separately for the 9 favourable and 7 unfavourable years, as presented in Table 4. Based on all 16 years, the MS values indicated that the effect of N fertiliser was the most important, followed by the year, the hybrid and the sowing date. Both the main effects and interactions of the N fertiliser, sowing date and hybrid were significant. Consequently the main effects of the treatments can only be interpreted to a limited extent. The N fertiliser × sowing date, N fertiliser × hybrid and sowing date × hybrid interactions are illustrated in Figure 1, based on the data of 16 years.

The N fertiliser response in the early and optimum sowing dates was significantly different from that of the late and very late sowings in the N fertiliser × sowing date interaction (Fig. 1). This was manifested in the higher yield level of the early and optimum sowings and in the fact that the yield only responded to higher N rates up to 60 kg ha^{-1} in the late and very late sowings, compared with significant yield increases up to 120 kg ha^{-1} N in the early and optimum sowings. At a rate of 240 kg ha^{-1} N there was a drop in yield, except in the early treatment. The linear effect was dominant in the N response, and was shown by the partition of the SS values to be more than four times as high as the quadratic effect. When the treatment groups were compared, the deviation was greatest between the N_0 treatment and the other N treatments. There was also a significant difference between N_{60} and the other treatments, and between N_{240} and the other treatments.

Table 4

Results of combined analysis of variance over the whole period of the experiment and in favourable (9) and unfavourable (7) years

Source of variation	1991–2006		Favourable years		Unfavourable years	
	df	MS	df	MS	df	MS
Year (Y)	15	773.6	8	269.5	6	955.2
Year (Repl.)	48	12.5	27	13.7	21	11.1
N fertiliser (N)	4	1192.4^{***}	4	80.2^{***}	4	296.5^{***}
Residual	252	11.1	140	12.5	108	6.5
Sowing date (S)	3	338.6^{***}	3	99.9^{***}	3	144.2^{***}
N × S	12	19.6^{***}	12	8.9^{***}	12	12.8^{***}
Residual	945	3.6	525	3.1	405	4.4
Hybrid (H)	3	666.2^{***}	3	65.0^{***}	3	306.2^{***}
N × H	12	8.8^{***}	12	3.3^{***}	12	4.8^{***}
S × H	9	4.0^{**}	9	1.8^{NS}	9	5.4^{***}
N × S × H	36	1.2^{NS}	36	<1	36	1.3^{NS}
Residual	3780	1.5	2100	1.4	1620	1.4

Significance levels: NS = not significant at $P < 0.05$, ** Significant at $P \leq 0.01$, *** Significant at $P \leq 0.001$ levels, respectively

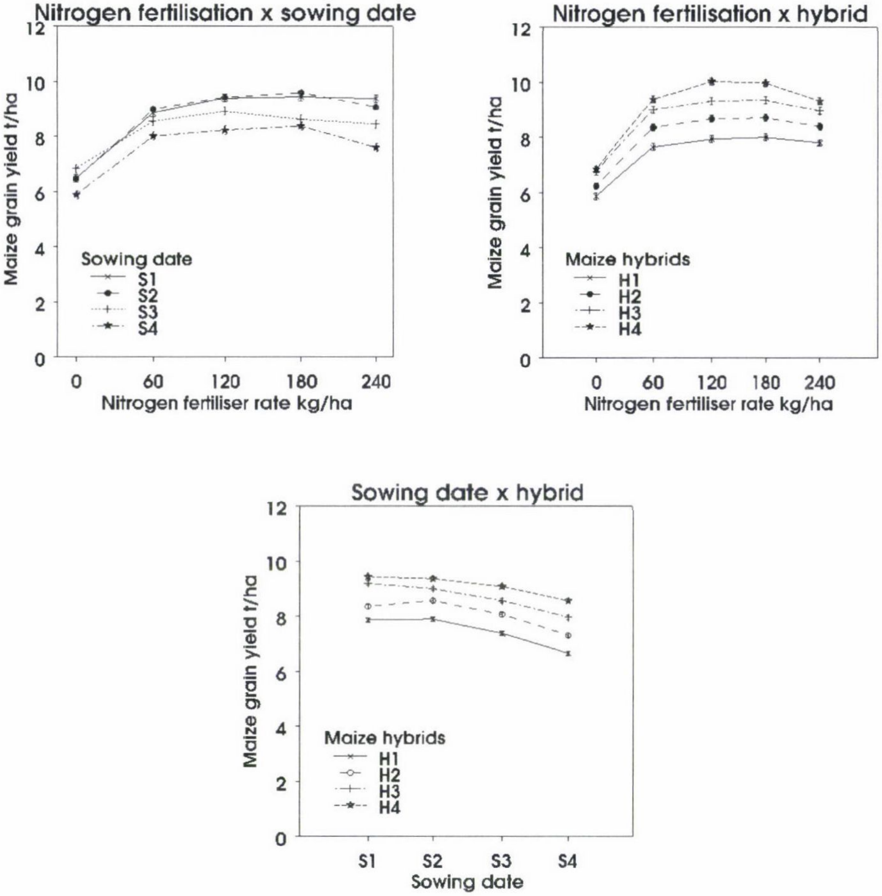

Fig. 1. Effect of interactions between N fertilisation and sowing date, N fertilisation and hybrid, and sowing date and hybrid on the grain yield of maize, averaged over 16 years. Vertical bars = ± S.E. LSD (P<0.05) values are for interactions

In the N fertiliser × hybrid interaction (Fig. 1) the N response of the latest maturing hybrid, H_4, differed from that of the other three hybrids. The N fertiliser response of the hybrids was characterised by a large significant yield increase at the N_{60} level, a slight but significant increase at the N_{120} level and a significant yield reduction at the N_{240} level. The H_4 hybrid differed from the others in that there was a steeper increase in yield between N_{60} and N_{120} and a greater decline between N_{180} and N_{240}. A comparison of the treatment groups showed the greatest difference between the control and the other N treatments. The difference in yield level between the hybrids exhibited a close correlation with the vegetation period. Both linear and quadratic effects were significant in the N responses of the hybrids, but the quadratic effect was more than three times as great as the linear effect.

An analysis of the sowing date × hybrid interaction (Fig. 1) revealed that there was no significant difference in the yield between the optimum and early sowing dates. In the late and very late sowings the yields of the hybrids declined significantly, but to an extent differing for each hybrid. Of the four hybrids investigated, the sowing date responses of H_1 and H_2 were similar, as were those of H_3 and H_4. In the sowing date × hybrid interaction both the linear and quadratic effects were significant and of similar magnitude.

When the year effect was considered (Table 4), the MS values indicated that in favourable years N fertilisation had the greatest effect, followed by the year, the hybrid and the sowing date, while in unfavourable years the year effect was greatest, followed by the hybrid, N fertilisation and the sowing date. Both the main effects of the treatments and the interactions were significant in all the years, but the MS values showed that the main effects substantially exceeded the interactions.

The yield-increasing effect of N fertilisation was greater in favourable years than in unfavourable years at all N levels (Fig. 2). In favourable years significant yield increases were recorded up to a rate of 120 kg N ha^{-1}, but in unfavourable years this was only true up to 60 kg N ha^{-1}. As an effect of the year, greater reductions in the yield were observed between N_{240} and N_{180} in unfavourable years than in favourable years (0.635 vs. 0.195 t ha^{-1}). In the sowing date treatments the year effect was manifested in the fact that a consistent decline in the yield was observed at all the sowing dates compared with the early treatment in unfavourable years, while in favourable years the yield decrease was only significant in the late and very late sowings. In both types of years, the yield of hybrids from later maturity groups surpassed that of hybrids with shorter vegetation periods.

Additive main effects and multiplicative interaction analysis of variance for grain yield

Additive main effects and multiplicative interaction analysis of variance partitioned the treatment SS into additive genotype (nitrogen, sowing date, hybrid) and environment (year) effects, and non-additive GE interaction (Y × N, Y × S, Y × H) effects. These sources were all significant at the 0.01 probability level. The proportion of sum of squares due to differences between years ranged from 62 to 84%, variation due to genotype from 7 to 26%, and variation due to G × E from 9 to 12% (Table 5). Note that the environment effect dominated the analysis. The MS values of ANOVA showed that N fertiliser had the greatest effect, followed by hybrid and sowing date effects. The F-test indicated that both principal components of the interaction (PCA1 and PCA2) were significant. These components explained a considerable proportion of the interaction SS values: 97% for year × N fertiliser, 92% for year × sowing date and 85% for year × hybrid.

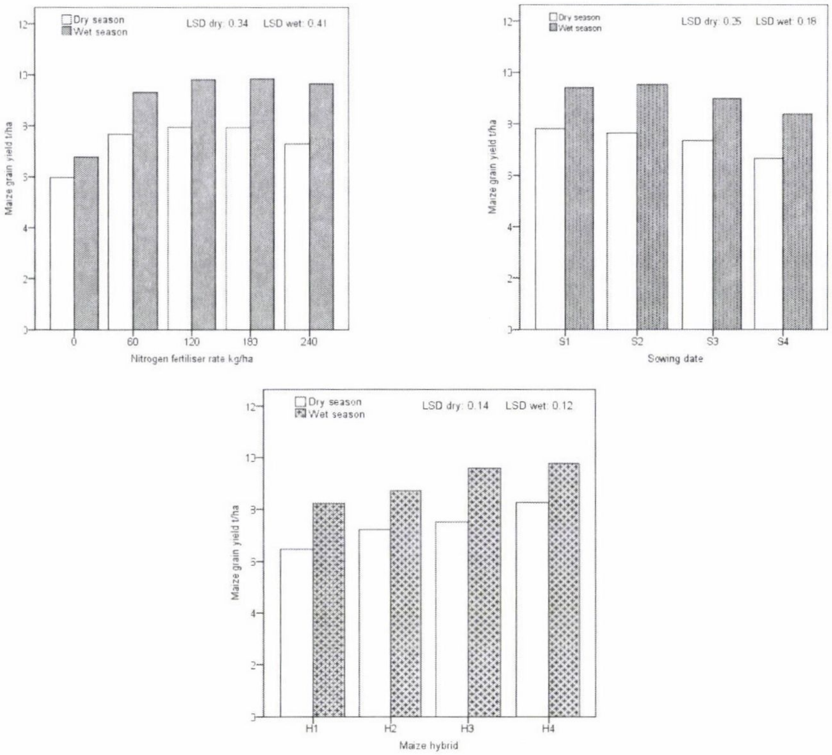

Fig. 2. Effect of N fertilisation, sowing date and hybrid on the grain yield of maize in dry (7) and wet (9) years. LSD ($P<0.05$) values are for dry and wet seasons

In the biplot (Fig. 3) main effect means are shown on the abscissa and PCA1 values as the ordinates. Genotypes (or environments) with large PCA1 scores (either positive or negative) have high interactions, whereas genotypes (or environments) with PCA1 scores near zero have small interactions. The N_0 treatment is distinctly separated from the other treatments and is characterized by the greatest PCA1 score and the smallest grain yield, indicating that the control treatment (N_0) contributed to the greatest extent to the N treatment × year interaction, followed by the N_{240}, N_{180} and N_{120} treatments, while the N_{60} treatment was the most stable. The N_{120} and N_{180} treatments gave the highest yield. There was no significant difference in yield between the N_{240} and N_{60} treatments. The environments showed much variability in both main effects and interactions (Fig. 3). It can be seen that the very late (S_4) and early (S_1) sowing dates made the greatest contribution to the sowing date × year interaction, while the optimum (S_2) and late (S_3) sowing dates were the most stable. The highest yields were recorded for the early (S_1) and optimum (S_2) sowing dates. Among the hybrids, H_1 and H_4 (FAO 200–299 and FAO 400–499) made the greatest contribution to the interaction, and H_2 and H_3 (FAO 300–399) the smallest. The H_3 and H_4 hybrids gave the highest grain yield of maize.

Table 5

Additive main effects and multiplicative interaction analysis of variance for grain yield (t ha^{-1}) for the three-factorial experiment (1991–2006) including the first two principal component axes (PCA1, PCA2) for the interactions

Source of variation	df	MS	F-values
Treatment combinations	79	234.5	90.2***
Year (Y)	5	773.6	61.8***
N fertiliser (N)	4	1192.4	458.3***
Y × N	60	35.8	13.8***
PCA1	18	108.5	41.7***
PCA2	16	8.6	3.29***
Residuals	26	2.4	<1
Error	4992	2.6	
Treatment combinations	63	220.1	62.5***
Year (Y)	5	773.6	61.8***
Sowing date (S)	3	338.6	96.1***
Y × S	45	27.8	7.9***
PCA1	17	46.4	13.2***
PCA2	15	24.0	6.8***
Residuals	13	7.8	2.2**
Error	5008	3.5	
Treatment combinations	63	241.4	74.2***
Year (Y)	5	773.6	61.8***
Hybrid (H)	3	666.2	204.7***
Y × H	45	35.7	11.0***
PCA1	17	57.5	17.7***
PCA2	15	25.5	7.8***
Residuals	13	19.0	5.8***
Error	5008	3.3	

Significance levels: ** Significant at $P \leq 0.01$, *** Significant at $P \leq 0.001$ levels, respectively

Stability analysis

The effect of N fertilisation, sowing date and hybrid on yield stability was studied by the variance and regression methods of stability analysis (Table 6). Among the N fertiliser treatments the control treatment, without N fertilisation, had the highest coefficient of variance (12.57), while the lowest values were recorded between N_{60} and N_{180} (2.65–3.99). A comparison of the sowing dates revealed the highest CV% values for the very late and early sowing dates (8.81 and 6.63) and the lowest for the optimum date or ten days later (4.49 and 4.47). Among the hybrids, the early (H_1) and late (H_4) hybrids had the greatest CV% values (9.42 and 7.83, respectively).

The values of ecovalence (W^2) and stability variance (σ^2) were lowest and non-significant for the optimum sowing date and ten days later. The control treatment without N fertilisation and the excessively high (240 kg ha^{-1}) N rate were responsible for the significant N × year interaction. The W^2 and σ^2 parameters were not significant at N rates of 60, 120 and 180 kg ha^{-1}. Among the maize hybrids the early (H_1) and late (H_4) hybrids made significant contributions to the interaction. The sowing date and N fertilisation treatments selected as the most favourable using Kang's (1993) yield stability parameter (YS), which is based on the mean yield response and stability, were the same as those selected by the W^2 and σ^2 parameters. The YS parameter found hybrids in the FAO 300–399 and FAO 400–499 maturity groups to be the most favourable.

A comparison of the various sowing dates revealed regression coefficients (b) with values greater than 1.0 for optimum sowing date and ten days later (1.033 and 1.095, respectively). The regression coefficients indicated that the 120 kg ha^{-1} N rate had the greatest stability (b = 1.084) of all the N fertiliser treatments. The low b value (0.452) recorded for the control treatment without N fertiliser indicated better adaptation to an unfavourable environment. The hybrid in the FAO 200–299 group (H_1) had an average response to environmental factors, while the H_3 and H_4 hybrids indicated better adaptation to favourable environment and the H_2 hybrid should best be grown in less favourable environments.

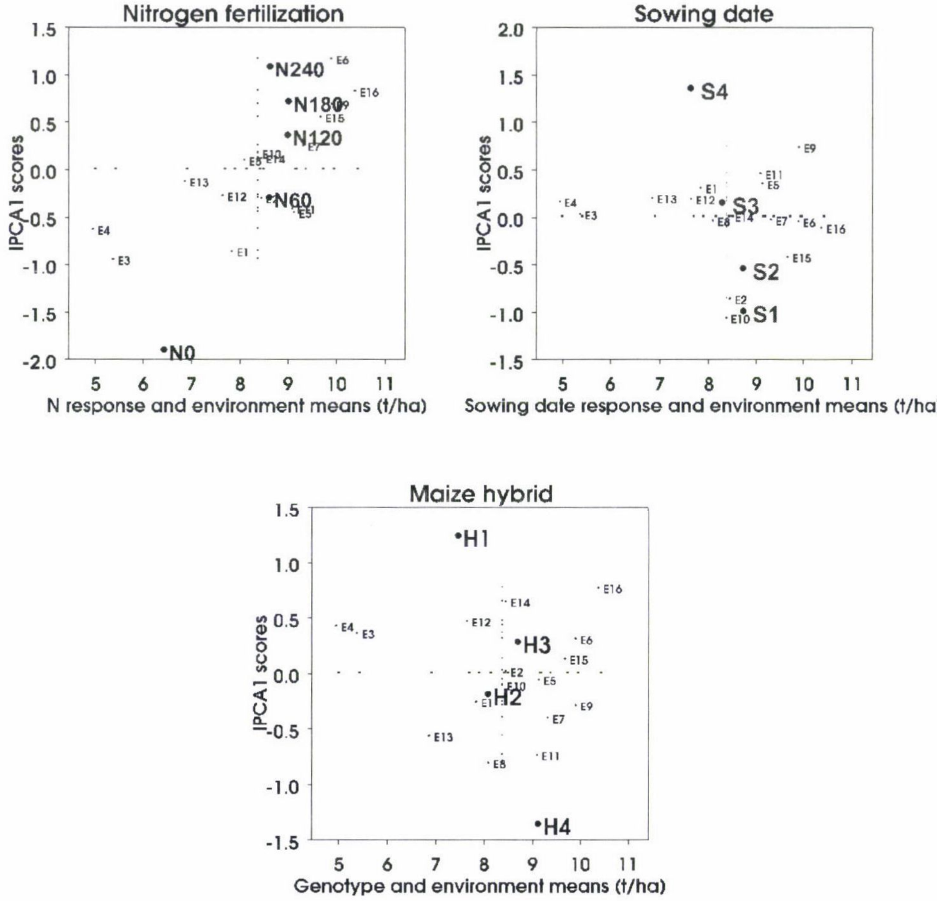

Fig. 3. Plot of mean yields and first principal component scores for N fertilisation, sowing date and maize hybrids in 16 environments

Table 6
Effect of sowing date, N fertilisation and hybrid on the yield and yield stability of maize (1991–2006)

Treatments	Yield response† [t ha^{-1}]	Variance parameters				Regression parameters‡		
		CV %	W^2	σ^2	YS§	r	b	a
N fertilisation (N)								
N_0	6.429c	12.57	80.18	8.16***		0.669**	0.452	2.665
N_{60}	8.599b	2.65	3.89	0.31^{NS}	+	0.988***	0.918	0.957
N_{120}	8.987a	3.43	6.34	0.42^{NS}	+	0.985***	1.084	–0.043
N_{180}	9.006a	3.99	13.19	0.72^{NS}	+	0.983***	1.203	–1.012
N_{240}	8.619b	5.72	30.87	2.68***		0.975***	1.344	–2.577
Sowing date (S)								
Early (S_1)	8.712a	6.63	19.18	1.86^{NS}	+	0.934***	0.942	0.868
Optimum (S_2)	8.706a	4.49	8.56	0.45^{NS}	+	0.974***	1.033	0.107
Late (S_3)	8.273b	4.47	8.98	0.50^{NS}	+	0.979***	1.095	–0.845
Very late (S_4)	7.621c	8.81	25.92	2.76***		0.913***	0.932	–0.137
Maize hybrid (H)								
H_1	7.452a	9.42	27.61	2.79***		0.917***	1.004	–0.909
H_2	8.074b	4.72	10.99	0.57^{NS}	+	0.964***	0.859	0.919
H_3	8.680c	5.29	12.13	0.72^{NS}	+	0.964***	1.045	–0.026
H_4	9.106d	7.83	29.78	3.08***	+	0.927***	1.092	0.010

CV: coefficient of variance; W^2: ecovalence; σ^2: stability variance; YS: yield stability; r: correlation coefficient; b: regression coefficient; a: regression constant. †: Within each factor, yields designated with the same letter do not differ significantly from each other according to Duncan's Multiple Range Test. NS = Non-significant at $P \leq 0.05$, **Significant at $P \leq 0.01$, ***Significant at $P \leq 0.001$ levels, respectively; §: + indicates the best treatments on the basis of yield stability; ‡: Number of data pairs (years) in the regression analysis: n = 16.

Effect of treatments on agronomic and ecophysiological characteristics of maize plants

As the result of the environmental changes associated with the sowing dates, there were significant differences in the silking dates (Table 7). The effects of sowing date and the maturity period of the hybrid were significant in all the years, and that of N fertiliser in all but one. The joint evaluation of the 16 years demonstrated that the effect of sowing date was the most important, being around 7 times that of the hybrid (based on MS values), while that of N fertilisation was only one fourteenth of the hybrid effect. Averaged over the experimental years, the date of silking (expressed as days from sowing) in the various sowing date treatments was as follows: early: 82.9, optimum: 76.5; late: 73.1; very late: 69.8. The number of days from sowing to silking was 73.0 for the hybrids in the earliest group, gradually increasing to 78.1 days in the latest group. In agreement with the findings of Nielsen et al. (2002), the sowing date × hybrid interaction was significant every year.

The effect of N fertilisation on the N supplies to maize was well reflected by the SPAD values, indicative of the chlorophyll content. Measurements made from 2001–2006 showed that the SPAD index was lowest in the control treatment, significantly increasing up to a rate of 180 kg ha^{-1} N. The following values were obtained in the various N treatments, averaged over the years: N_0: 44.8, N_{60}: 52.4, N_{120}: 56.3, N_{180}: 57.8, N_{240}: 58.0. There was a significant correlation between grain yield and the SPAD values ($r = 0.679^{***}$, n = 30).

The sowing date and N fertilisation had a significant effect on the net photosynthesis rate of maize in all the years examined (Table 7). The rate of photosynthesis was greatest after sowing at the optimum date or within ten days of this, while it was lowest after very late sowing. Compared with the N_0 control, N fertilisation led to an increase in the rate of photosynthesis, but in general there was no significant difference between the N treatments. A correlation significant at the 0.1% level was observed between the photosynthesis rate and the yield, averaged over the maize hybrids (r = 0.680, n =36).

Table 7

Effect of N fertilisation, sowing date and hybrid on some agronomic and ecophysiological characteristics of maize plants

Treatments	Silking date (days from sowing)	Photosynthetic rate (μmol $m^{-2} s^{-1}$)	Chlorophyll content (SPAD values)	Grain moisture (%) at harvest	Grain number m^{-2}	1000-kernel mass (g)
N fertilisation (N)						
N_0	76.7	20.68	44.8	20.01	1825	293.2
N_{60}	75.3	24.71	52.4	19.73	2561	309.6
N_{120}	75.1	25.43	56.3	19.84	2719	320.5
N_{180}	75.3	25.40	57.8	19.72	2707	321.4
N_{240}	75.5	25.16	58.0	19.69	2711	321.5
LSD (0.05)	0.27	0.64	1.40	0.18	149	7.2
Sowing date (S)						
Early (S_1)	82.9	24.75	50.9	17.93	2402	314.9
Optimum (S_2)	76.5	25.83	53.1	18.46	2407	322.6
Late (S_3)	73.1	24.37	55.4	20.09	2430	309.4
Very late (S_4)	69.8	22.15	56.0	22.70	2354	306.1
LSD (0.05)	0.47	0.50	0.70	0.16	60	4.0
Maize hybrid (H)						
H_1	73.0	24.80	55.8	18.41	2459	291.7
H_2	74.9	–	52.3	19.80	2237	322.2
H_3	76.2	24.83	53.0	20.66	2433	309.7
H_4	78.1	23.19	53.4	20.32	2465	329.3
LSD (0.05)	0.14	0.44	0.44	0.16	44	3.3
F-test results						
N	***	***	***	**	***	***
S	***	***	***	***	NS	***
H	***	***	***	***	***	***
N × S	NS	*	**	***	NS	NS
N × H	NS	*	NS	**	NS	***
S × H	***	***	*	***	**	**
N × S × H	NS	NS	NS	NS	NS	NS

Silking date and grain moisture data are for 1991–2006, photosynthetic rate and SPAD value data for 2001–2006, yield component data for 2001–2006. NS = not significant, * Significant at $P \leq 0.05$, ** Significant at $P \leq 0.01$, *** Significant at $P \leq 0.001$

Among the yield components, the thousand-kernel mass was significantly influenced by the sowing date in all the years, being highest in the optimum and early sowings and decreasing significantly in the late and very late sowings. The effect of sowing date on the number of kernels per m² varied according to the year, being significantly lower in the very late sowing, averaged over 6 years, while there was no significant change in the other sowing dates. N fertilisation had the greatest effect on the number of kernels per m². In the N_0 treatment this was only 70% as great as in the N_{60} treatment. A further increase was observed at N_{120}, after which no significant change was recorded. The thousand-kernel mass was smallest in the N_0 control and significantly greater in the N_{60} and N_{120} treatments, after which the changes were not significant (Table 7).

In each year the effect of the treatments on the grain moisture content at harvest was recorded. The effect of the hybrid had a significant effect on the grain moisture content every year, and the sowing date in all but two years. The effect of N fertilisation was significant in six years. A joint evaluation of the 16 years showed that the effect of the sowing date was more than four times that of the hybrid. As the result of sowing date the grain moisture content at harvest was lowest in the early (17.9%) and optimum (18.5%) sowings and substantially greater in the late (20.2%) and very late (22.7%) sowings. Significant differences were observed for the grain moisture contents of the individual hybrids, the later hybrids tending to have higher values than the early ones (from early to very late: 18.4, 19.8, 20.7 and 20.3%). Among the interactions, the sowing date × hybrid interaction was the most important, due to the major increase in the grain moisture content of later-maturing hybrids when sown late or very late, compared with hybrids with shorter vegetation periods.

Discussion

The agronomic factors studied in the long-term N fertility experiment had a great influence on the amount of resources available for maize growth and grain yield formation and on the resource use efficiency determining both the final yield and yield stability. The sowing date had a significant effect on the maize yield in all the 16 years examined. The highest maize yields were obtained for the early and optimum sowing dates. Early and intermediate sowings tend to best utilize solar radiation for grain production (Otegui et al., 1995; Cirilo and Andrade, 1996). Compared to the optimum sowing date, yield reductions of 5% and 12.5% were recorded, averaged over the years, when sowing was delayed by ten and twenty days, respectively. In some years, however, the magnitude of the yield reduction was considerably greater (e.g. in 1992 and 2000, when the yield dropped by 30% and 40%, respectively, in the very late treatment). Calendar time from sowing to silking decreased by about 13 days when maize was planted in mid-May compared with early April. Delayed sowing shortens the effective growing season for maize, increasing the risk of exposure to lethal cold temperatures late in the season before grain maturation (Nielsen et al., 2002; Sárvári, 2005). The effect of sowing date and N fertilisation was clearly reflected in the efficiency of photosynthesis at flowering, in agreement with earlier results (Berzsenyi et al., 2006).

Ding et al. (2005) showed that the difference in dry matter accumulation between maize grown under high and low N conditions was associated with sustained higher leaf carbon exchange rate (CER) and chlorophyll content during grain-filling and that response to N availability was consistent for a set of older and newer maize hybrids. Echarte et al. (2008) also found that at the leaf level, leaf CER declined during the grain-filling period, and the decline was greater under low than under high N availability. Delays in sowing significantly reduced the thousand-kernel mass in all the years, being highest in the optimum and early sowings and decreasing significantly in the late and very late sowings. Studying the sowing date effects on grain yield components for different maize genotypes Otegui et al. (1995) recognized different genotype responses. While the grain yield of prolific hybrids was responsive only to grain number per square metre, that of non-prolific ones also changed with grain weight. In the present experiment the hybrids examined were non-prolific and showed similar responses.

The effect of N fertilisation was significant in all years but one, and surpassed the effect of sowing date with the exception of two years. In the late and very late sowings and in years with unfavourable weather conditions, yield increments were only observed up to an N rate of 60 kg ha^{-1}, while in the early and optimum sowings and in favourable years yield increments were significant up to 120 kg ha^{-1} N. In agreement with the findings of Schepers et al. (1992), the chlorophyll index (SPAD values) gave a good indication of the N status of the maize plants over the years of the study. Echarte et al. (2008) showed that reductions in leaf CER associated with low N availability were accompanied by variation in the chlorophyll index, and consequently, leaf absorbance. Among the yield components, N fertilisation had the greatest effect on the number of kernels per m^2. The thousand-kernel mass was smallest in the N_0 control and significantly greater in the N_{60} and N_{120} treatments, after which the changes were not significant. In both favourable and unfavourable years the yields of hybrids with longer vegetation periods surpassed those of hybrids in earlier maturity groups. The grain moisture content at harvest was influenced to the greatest extent by the sowing date. It could be concluded from the sowing date × hybrid interaction, that the grain moisture content of hybrids with longer vegetation periods increased to a greater extent when sown late or very late than that of hybrids with shorter growing periods.

Yield stability depends on yield components and other plant traits, such as pest resistance, stress tolerance and environmental variables. By identifying and regulating the most limiting factors, stability can be substantially improved (Kang and Gauch, 1996). Under the given experimental conditions, the environmental variable with the greatest influence on yield stability was the amount and distribution of rainfall during the growing period. All the agronomic factors tested (sowing date, N fertilisation and genotype) had a great influence on yield and yield stability. It is thus important to identify the response pattern of the factors with the best yield stability for recommendation to farmers. In agreement with Tollenaar and Wu (1999) it could be concluded that high yields and yield stability are not mutually exclusive. Both multivariate and univariate stability analysis were able to give an indication of the stability of genotypes and cropping systems. Their results were in agreement and complemented each other.

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RESULTS OF A 30-YEAR-OLD FERTILISATION EXPERIMENT

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The long-term mineral fertilisation experiment set up on acidic brown forest soil in Gödöllő in 1972 has made it possible over the years to answer a number of questions. It became clear that, in general, increasing rates of NPK fertilisation only caused a significant increase in the yields obtained in the crop rotation at lower rates (150–300 kg NPK/ha). As time went on, rates higher than this caused yield depression. The continuous application of high fertiliser rates led to a substantial increase in the P and K contents of the ploughed layer and in the quantity of nitrate accumulating in the 3 m soil profile. An increase in the quantity of nutrients was associated with a reduction in the pH and in the content of Ca and Mg. When mineral fertilisation was omitted for six years, there was a substantial reduction in the P and K contents of the ploughed layer.

Key words: fertilisation, yield, quality, liming, manuring

Introduction

Many mineral fertilisation trials were set up at various locations in Hungary from the mid-1960s, some of which formed part of a uniform experimental network, while others represented an independent attempt to find answers to the problems raised by the new practice of mineral fertilisation. In most cases these were not originally intended to be long-term experiments, but were designed to solve specific problems facing farmers. These field trials on mineral fertilisation and crop production, which gradually became long-term experiments, were modified over the years to provide answers to new problems The results of field trials were recently summarized in a number of publications (Csathó, 2004; Debreczeni and Debreczeniné, 1994; Kádár, 1992; Kádár and Szemes, 1994; Kovács and Füleky, 1991).

Field trials on mineral fertilisation set up by the Gödöllő University of Agricultural Sciences in 1972 at an experimental nursery in Szárítópuszta aimed to investigate the following questions:

1st period: 1972–1980

What influence do intensive fertiliser application and plant protection have on the quantity and quality of yield?

2nd period: 1980–1986

How are the quantity and quality of yield and the properties of the soil influenced by annual fertiliser application, the omission of fertilisation, and the application in advance of higher rates intended for a 3-year period?

3rd period: 1986–1990

How can the deterioration in soil properties be ameliorated by liming and organic manuring, and how does this affect the yield?

4th period: 1990–1998

How can intensive, semi-intensive and non-intensive land use be applied on soils with various nutrient supply levels?

5th period: from 1998

What effect does the nutrient status of the soil have on the plantation of agroforests?

Materials and methods

The Szárítópuszta nursery of the University of Agricultural Sciences is situated in the Gödöllő hills some 30 km north of Budapest. The soil is brown forest soil with a sandy loam texture, acidic pH (pH_{KCl} 5.0), OM content 1.5%, and poor nutrient supplies (AL-P_2O_5 = 34 mg kg^{-1}, AL-K_2O = 129 mg kg^{-1}).

1st period

The field experiment was set up at the Szárítópuszta nursery in 1972. During the first eight years three crop rotations (A, B, C) were planted, involving winter wheat, maize, potatoes, sugar beet and sunflower. Each rotation consisted of three blocks (a, b, c), involving various weed control treatments: a) mechanical control, no chemicals; b) moderate levels of chemical control; c) intensive chemical control. The five fertiliser rates (0, 150, 300, 600, 900 kg N, P_2O_5, K_2O/ha/year), applied as ammonium nitrate, superphosphate and potassium chloride, were maintained in practice throughout the long-term trial. The active agent ratio of the three nutrients over the crop rotation averaged 1.7 : 1 : 1.3. The plot size was 70 m^2, with four replications of each treatment (I–IV). Regular measurements were made on the grain and by-product yields and on the N, P and K contents of the yield. In 1980 the soil in the ploughed layer was analysed in each plot for the whole experiment.

2nd period

During the six years after 1980 only minor changes were made in the experiments. The highest fertiliser rates were reduced from 600 to 450 kg and from 900 to 600 kg, and this level was maintained in later stages. The weed control treatments were discontinued, being replaced by fertiliser use blocks:

a) annual mineral fertilisation,
b) analysis of residual effect,
c) PK fertilisation for 3 years in advance + annual N fertilisation.

The main and by-product yields and the N, P, K, Ca, Mg, Fe, Mn, Zn and Cu contents of the yield were regularly recorded. Soil analysis, involving the AL-P and K contents, pH, organic matter and microelement contents of the soil, was carried out in 1983 and 1986. In 1986 the depth distribution of nitrate in the soil was also recorded.

3rd period

After 1986 it was necessary to ameliorate soil properties such as pH and humus content that had deteriorated. The differences in fertiliser use (a, b, c) were discontinued, the five annual levels of fertilisation (M) remained, and the earlier replications (I, II, III, IV) were replaced by the following treatments:

I. Original mineral fertilisation (M + m), II. Original mineral fertilisation (M) + farmyard manure (#), III. Original mineral fertilisation (M + m) + liming (Ca), IV. Original mineral fertilisation (M) + farmyard manure (#) + liming (Ca). In treatments to which farmyard manure was not applied, mineral fertilisers (m) containing nutrient contents identical to those supplied by the uniform rate of farmyard manure (#) were additionally applied.

The annual lime rate averaged 1.5 t $CaCO_3$ ha^{-1} as powdered limestone. Farmyard manure was applied every three years, in a quantity of 30 t ha^{-1}. The main and by-product yields were recorded each year, and detailed soil analysis was carried out in 1989.

4th period

From 1990 to 1998 the experiment was continued as follows: Intensive mineral fertilisation was continued in crop rotation A, with farmyard manure every 3 years and liming. The crop sequence in these years was: maize, wheat, maize, wheat, maize, wheat, maize. The main and by-product yields were recorded each year, and detailed soil analysis was carried out in 1998.

In crop rotation B semi-intensive land use was applied. The previous rates of mineral fertiliser, farmyard manure and lime were continued until 1992, after which no further fertiliser was applied. The crop sequence was: maize, rye, oats, alfalfa, alfalfa, barley. The main and by-product yields were recorded each year, and detailed soil analysis was carried out in 1998.

In crop rotation C the previous rates of mineral fertiliser, farmyard manure and lime were continued until 1990, after which no further fertiliser was applied. The crop sequence was: maize, grass, grass, grass, grass, barley. The main and by-product yields were recorded each year, and detailed soil analysis was carried out in 1998.

5th period

In 1999 oak saplings were planted on the site of crop rotation A, while crop rotations B and C were discontinued.

Results and discussion

In the case of winter wheat the tendency for only the lowest, 150 kg/ha NPK fertiliser rate to increase the yield, while higher rates led to yield depression, was observed even during the 1st period (Table 1). A moderate rate of chemical weed control resulted in significantly lower yields than on plots where no chemicals were applied. The situation was somewhat different for maize. Higher fertiliser rates continued to improve the yield, though to a lesser extent. The highest yield of maize was achieved with intensive weed control. A similar picture was observed for potato and sugar beet, though in the latter crop the highest yield was recorded with the moderate rate of chemical weed control.

In the 2nd period the lowest yields of winter wheat, maize and potato were obtained in the residual effect treatment, i.e. when no further fertiliser was applied (Table 2).

Intensive mineral fertilisation and fertilisation in advance for three years had much the same effect. In the case of winter wheat the yield was only significantly increased by the first (150 kg/ha) and in some cases the second (300 kg/ha) rate of NPK.

Table 1

Effect of weed control and increasing rates of mineral fertiliser in a long-term experiment carried out on brown forest soil in Gödöllő, averaged over the years 1973–1980 and over factors

Factor	Weed control	Fertiliser active agent levels kg/ha/year					Mean	$LSD_{5\%}$
		0	150	300	600	900		
For the grain yield of winter wheat	Block a	4.11	4.81	4.63	4.15	4.12	4.37	0.31
	Block b	3.84	4.39	4.29	3.78	3.48	3.95	
	Block c	4.10	4.43	4.43	3.91	3.64	4.10	
	Mean	4.02	4.54	4.45	3.95	3.75	4.14	
	$LSD_{5\%}$			0.24				
For the grain yield of maize	Block a	4.75	5.03	5.16	5.09	5.05	5.02	0.21
	Block b	5.35	5.60	5.52	5.55	5.61	5.53	
	Block c	5.55	5.63	5.83	5.85	5.76	5.73	
	Mean	5.22	5.42	5.51	5.50	5.47	5.43	
	$LSD_{5\%}$			0.26				
For the tuber yield of potato	Block a	9.97	12.06	13.41	15.67	16.05	13.43	0.99
	Block b	12.33	15.47	16.48	18.09	17.55	15.99	
	Block c	13.17	14.87	17.14	18.30	18.36	16.37	
	Mean	11.82	14.13	15.67	17.00	17.32	15.26	
	$LSD_{5\%}$			1.28				
For the root yield of sugar beet	Block a	28.74	32.27	32.11	33.80	35.52	32.49	2.97
	Block b	34.16	38.29	38.97	42.60	42.20	39.26	
	Block c	32.46	35.50	36.45	40.33	36.97	36.97	
	Mean	31.79	35.38	35.84	38.84	39.35	36.24	
	$LSD_{5\%}$			3.86				

Blocks: a = mechanical control; b = moderate level of chemical control; c = intensive chemical control

Table 2

Effect of fertiliser rates and the mode of application in a long-term experiment on brown forest soil in Gödöllő, averaged over the years 1981–1986 and over factors

Factor	Weed control	Fertiliser active agent levels kg/ha/year					Mean	$LSD_{5\%}$
		0	150	300	600	900		
For the grain yield of winter wheat	Block a	3.79	5.17	5.45	5.37	5.05	4.97	0.26
	Block b	3.57	4.11	4.34	4.69	5.11	4.37	
	Block c	3.72	5.12	5.33	5.18	5.01	4.87	
	Mean	3.69	4.80	5.05	5.08	5.06	4.74	
	$LSD_{5\%}$			0.34				
For the grain yield of maize	Block a	5.49	6.28	6.22	5.72	5.37	5.82	0.32
	Block b	5.17	5.22	5.46	5.63	5.22	5.34	
	Block c	5.21	5.86	5.87	5.29	4.56	5.36	
	Mean	5.29	5.79	5.85	5.55	5.05	5.50	
	$LSD_{5\%}$			0.41				
For the tuber yield of potato	Block a	15.66	20.19	20.99	22.24	22.33	20.28	1.33
	Block b	14.81	16.24	16.21	19.12	19.49	17.17	
	Block c	15.42	20.37	21.81	23.70	22.38	20.74	
	Mean	15.30	18.93	19.67	21.69	21.40	19.40	
	$LSD_{5\%}$			1.71				

Blocks: a = annual mineral fertilisation; b = residual effect; c = PK fertilisation for 3 years in advance + annual N fertilisation

The effect of increasing fertiliser rates was similar for maize, except that here yield depression was observed at fertiliser rates of 450 and 600 kg/ha. For potato the greatest yield increase was obtained with the lowest fertiliser rate (150 kg/ha), while higher fertiliser rates resulted in slight increases in the yield. During the 2nd period an analysis was also made of the macro- and microelement contents (Table 3). The macroelement content was generally increased by higher rates of mineral fertiliser. It was surprising to note that on this loose-textured, non-calcareous, acidic soil the Mn content of wheat grain and straw increased substantially as the result of higher fertiliser rates, while the discontinuation of fertilisation (Block b) reduced the Mn content in both the grain and the straw.

In addition to analysing the chemical content of the plants, changes in soil properties were also investigated (Table 4).

The regular application of high rates of fertiliser caused a substantial reduction in soil pH. The greater the annual fertiliser rate, the greater the reduction in pH. When fertilisation was omitted for 3 or 6 years, the extent of soil acidification declined (Füleky, 1997). This reduction in pH as the result of higher rates of intensive fertilisation could be attributed to a sharp drop in the Ca and Mg contents of the soil. By 1986 the Ca content in soil receiving the highest fertiliser rate had dropped to half compared with that in the control plot. This was accompanied by a decline in the soil Mg content, while the Mn content increased slightly. Annual high rates of mineral fertiliser led to a substantial rise in the AL-soluble phosphorus and potassium contents of the soil, while the omission of fertilisation between 1980 and 1986 led to a considerable reduction in the soil P and K contents.

Both liming (Ca) and farmyard-manuring (#) had a favourable effect on the winter wheat grain yield in the 3rd period between 1986 and 1990 (Table 5), but did not result in a significant increase in the yields of maize, potato or soybean. It should be noted, however, that the supplementation of the mineral fertiliser rates with a quantity equivalent to the nutrients in farmyard manure had a considerable effect on the yield for several years.

Table 3

Effect of continuous mineral fertilisation (1973–1980) and its temporary suspension (1981–1986) on the manganese content of wheat, averaged over the years 1981–1986, Mn mg kg^{-1}

Mode of fertiliser application	Fertiliser active agent levels kg/ha/year					$LSD_{5\%}$
	0	150	300	450	600	
Block a grain	40.5	41.4	48.0	55.1	60.8	6.4
Block b grain	40.4	41.9	42.1	48.0	50.1	
Block a straw	81.1	82.8	100.4	135.5	110.0	10.5
Block b straw	72.1	81.7	86.8	120.2	104.4	

Blocks: a, b, see Table 2.

Table 4
Effects of long-term mineral fertilisation on the soil properties in Gödöllő

Factor	Year	Treatment	Fertiliser active agent levels kg/ha/year					$LSD_{5\%}$
			0	150	300	600	900	
On the pH_{KCl}	1980	Mineral fert.	4.7	4.6	4.4	4.1	4.1	0.5
	1983	Mineral fert.	4.8	4.4	4.2	4.1	3.9	0.4
		Residual effect	4.7	4.5	4.4	4.4	4.5	
	1986	Mineral fert.	4.3	4.1	3.9	3.8	3.7	0.2
		Residual effect	4.3	4.1	4.1	3.9	3.8	
On the AL-soluble Ca, Mg and Mn contents mg/kg	1986	Mineral fert., Ca	1045	828	766	649	489	130
	1986	Mineral fert., Mg	163	126	116	111	110	26
	1986	Mineral fert., Mn	155	158	163	164	168	36
On the AL-soluble P_2O_5 content mg/kg	1980	Mineral fert.	37	55	63	113	129	29
	1983	Mineral fert.	40	67	91	162	196	23
		Residual effect	41	47	68	114	171	
	1986	Mineral fert.	37	82	120	194	267	14
		Residual effect	32	37	50	92	135	
On the AL-soluble K_2O content mg/kg	1980	Mineral fert.	124	144	167	206	200	48
	1983	Mineral fert.	123	140	145	216	228	25
		Residual effect	112	127	140	171	209	
	1986	Mineral fert.	118	126	153	200	250	12
		Residual effect	108	119	119	155	191	

Table 5
Effect of organic and mineral fertilisation and liming to brown forest soil in Gödöllő

Factor	Fertiliser variants	Fertiliser active agent levels kg/ha/year					Mean	$LSD_{5\%}$
		0	150	300	600	900		
On the grain yield of winter wheat, t/ha (1988)	M+m	4.19	7.09	6.92	6.23	5.37	5.96	0.40
	M+#	4.66	6.95	7.57	7.04	5.51	6.35	
	M+m+Ca	5.24	6.66	7.14	6.94	6.54	6.50	
	M+#+Ca	(4.28)	6.58	7.34	6.95	6.03	6.24	
	Mean	4.59	6.82	7.24	6.79	5.86	6.26	
	$LSD_{5\%}$			0.45				
On the grain yield of maize (1990)	M+m	3.28	3.54	3.21	2.60	2.77	3.08	0.24
	M+#	2.86	3.41	3.23	2.98	2.45	2.98	
	M+m+Ca	3.55	3.43	3.13	2.80	2.76	3.14	
	M+#+Ca	3.44	3.17	2.63	2.75	2.34	2.84	
	Mean	3.28	3.39	3.05	2.78	2.58	3.02	
	$LSD_{5\%}$			0.27				
On the tuber yield of potato t/ha (1987)	M+m	8.02	9.20	9.92	9.62	9.92	9.33	0.71
	M+#	7.53	9.82	10.54	9.66	10.26	9.55	
	M+m+Ca	7.94	8.92	9.71	11.41	10.31	9.66	
	M+#+Ca	7.59	9.68	9.76	10.62	10.48	9.63	
	Mean	7.77	9.41	9.98	10.33	10.24	9.55	
	$LSD_{5\%}$			0.80				
On the bean yield of soybean t/ha (1989)	M+m	0.85	1.09	1.30	1.35	1.22	1.16	0.08
	M+#	0.89	1.14	1.25	1.28	1.18	1.14	
	M+m+Ca	0.80	0.99	0.96	1.01	1.01	0.95	
	M+#+Ca	0.75	0.83	0.97	1.04	0.99	0.92	
	Mean	0.82	1.01	1.12	1.17	1.10	1.04	
	$LSD_{5\%}$			0.09				

M: Mineral fertiliser; m: mineral fertiliser equivalent to nutrients in farmyard manure; #: farmyard manure; figures in brackets indicate systematic soil error

By this time higher rates of mineral fertilisation were causing significant yield depression in winter wheat and maize.

During this period the effect of liming and organic manure on soil properties was also investigated. Liming was found to increase soil pH, while the application of organic manure increased the AL-soluble phosphorus and potassium contents of the soil (Table 6).

The nitrate-N quantities accumulating in the 3 m soil layer as the result of 14 years of mineral fertilisation were analysed in the various treatments (Table 7).

It is clear from the data that there was virtually no nitrate-N in the 3 m layer of soil that received no fertiliser. At moderate rates of nitrogen fertiliser a total of 787 kg nitrate-N was accumulated in the 3 m soil layer, while at high rates this figure was 2000 kg (1977). In general, less nitrate-N was found in the 0–60 cm soil layer, due to intensive plant nutrient uptake.

For the fourth crop production period (IV) the effect of continuous and discontinued fertilisation on the yield is presented from 1992. In the crop rotation intensively fertilised until 1993 (A) higher fertiliser rates caused yield depression in maize (Fig. 1). In the semi-intensive rotation (B) alfalfa was able to make good use of the nutrients accumulated in the soil. The same can be said of the hay yield in the extensive rotation (C) (Tasi and Füleky, 1998).

Table 6
Soil analysis data for the long-term fertilisation experiment in Gödöllő
(Crop rotations A, B and C, 1989)

Factor	Fertiliser variants	Fertiliser active agent levels kg/ha/year					Mean	$LSD_{5\%}$
		0	150	300	600	900		
	M+m	4.9	4.3	4.3	4.0	3.8	4.3	
	M+#	4.7	4.0	4.2	4.3	3.8	4.2	
pH_{KCl}	M+m+Ca	5.0	4.7	5.0	4.4	4.6	4.8	0.2
	M+#+Ca	5.2	5.2	5.3	4.9	4.8	5.1	
	Mean	5.0	4.6	4.7	4.4	4.3		
	$LSD_{5\%}$			0.3				
	M+m	45	94	104	182	194	124	
	M+#	43	121	144	180	241	146	
$AL-P_2O_5$	M+m+Ca	35	85	118	169	225	126	21
mg/kg	M+#+Ca	63	104	126	208	263	153	
	Mean	46	101	123	185	231		
	$LSD_{5\%}$			24				
	M+m	133	184	178	227	259	196	
	M+#	160	195	222	248	251	215	
$AL-K_2O$	M+m+Ca	156	168	214	224	205	194	
mg/kg	M+#+Ca	186	187	200	266	238	215	18
	Mean	159	184	203	241	238		
	$LSD_{5\%}$			20				

M: Mineral fertiliser; m: mineral fertiliser equivalent to nutrients in farmyard manure; #: farmyard manure

Table 7

Effect of long-term mineral fertilisation on the quantity of NO_3–N accumulated in the 3 m soil layer, kg/ha

Depth of the soil layer, cm	Fertiliser N applied, kg/ha/14 years			$LSD_{5\%}$
	0	1800	4600	
	Annual rate of fertiliser N kg/ha			
	0	130	330	
0–20	18	27	85	21
20–40	5	18	44	
40–60	3	22	40	
60–80	7	51	161	
80–100	0	60	151	
100–120	3	60	126	
120–140	5	65	153	
140–160	6	85	231	
160–180	6	66	206	
180–200	3	56	120	
200–220	5	61	145	
220–240	1	67	148	
240–260	10	63	121	
260–280	8	50	121	
280–300	7	35	121	
Total	87	787	1973	
$LSD_{5\%}$		12		

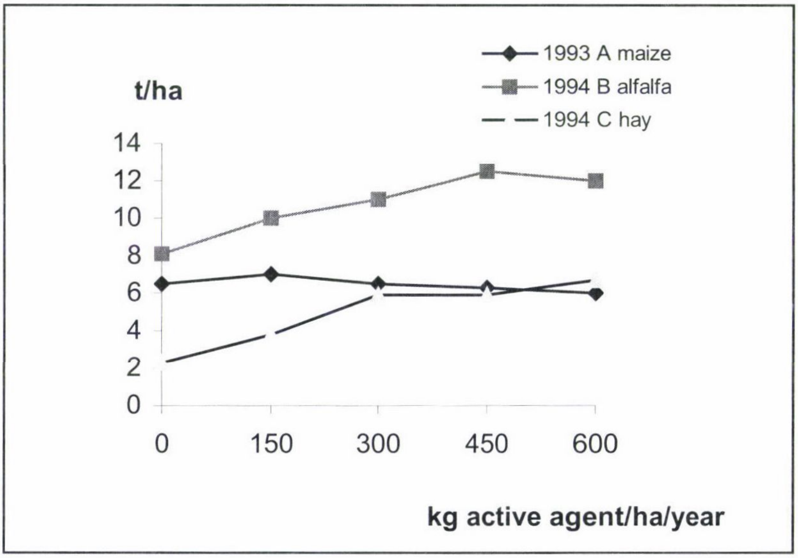

Fig. 1. Effects of intensive (A), semi-intensive (B) and non-intensive (C) land use on the yield

Conclusions

The fertilisation experiment, continued for over 30 years with almost the same rates of mineral fertiliser, was used by scientists to obtain answers to different questions in each period. During the 1st period it was found that chemical weed control led to somewhat higher yields than mechanical protection. Intensive fertilisation with high rates of fertiliser did not achieve its purpose at any stage during the 30 years. In general a substantial improvement in yields compared with the control plots was only achieved at a fertiliser active agent rate of 150 kg/ha.

When mineral fertilisation was discontinued for various lengths of time there was a drop in yield and a considerable decline in the phosphorus and potassium contents of the soil. Intensive fertilisation led to a reduction in soil pH, but improved the soil P and K contents. The further acidification of the soil could be prevented by liming. Organic manuring principally increased the P and K supplies of the soil. The analysis of nitrate-N to a depth of 3 m indicated that substantial quantities of nitrate-N were accumulated in the soil when N fertilisation exceeded the needs of the crop.

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IMPROVING EMERGENCE OF SOYBEAN (*Glycine max*) WITH STRAW MULCH AND OTHER PRACTICES UNDER NORMAL AND CRUSTED SOIL CONDITIONS

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Poor emergence is a major problem in soybean [*Glycine max* (L.) Merrill]. A high emergence count leading to an optimum plant stand is a pre-requisite to obtain high grain yields. The results of three experiments on emergence in soybean are reported in this paper. Two field experiments were conducted to study the influence of wheat straw mulch, farmyard manure (FYM) mulch and seed soaking in water on the emergence of soybean under normal conditions. Immediately after sowing, covering rows of soybean with 3 t wheat straw ha^{-1} (row mulch) or the whole plot with 6 t wheat straw ha^{-1} (plot mulch) showed a profound effect on improving both the speed of emergence and the final emergence count. Row mulch with 5 t FYM ha^{-1} only improved emergence in one year and its effect was not as great as with straw mulch. Soaking the seeds in water (on-farm seed priming) for 4, 8 or 16 h reduced emergence drastically. Another field experiment was conducted to study the effect of different mulching treatments or crust breaking in alleviating the crust effect of simulated rain on the emergence of soybean. Covering the soybean rows with 3 t wheat straw ha^{-1} (row mulch) either before or after simulated rain improved the emergence count. Covering the soybean rows with 5 t FYM ha^{-1} or breaking the crust also improved emergence, though the effects were not as good as with wheat straw mulch. The results suggest that the use of wheat straw mulch or FYM mulch can improve emergence in soybean in both normal and crusted soils, possibly by lowering the maximum soil temperature and conserving soil moisture.

Key words: crust formation, emergence, mulch, seed priming, soybean, wheat straw

Introduction

Soybean [*Glycine max* (L.) Merrill] is an important oilseed crop in India. It is grown from June to October; the sowing period extends from the beginning of June to the end of July. In rainfed areas sowing is done in July with the onset of the monsoon rains. However, rains before the emergence of the crop result in crust formation, leading to poor emergence. Therefore, under irrigated conditions efforts are made to sow soybean in June, when the likelihood of rain

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is low. However, during June temperatures are very high. It is generally recommended to sow soybean at a depth of 2.5–5.0 cm (Anonymous, 1999). Seeding too deep results in poor emergence, thus giving poor plant stands and consequently low grain yields. Shallow seeding results in poor emergence because of the fast drying of the surface soil due to the prevailing high temperature. One way to maintain low temperature and conserve moisture for a longer period is to cover the soil surface with mulching materials such as crop straw, farmyard manure, etc.

Straw mulch application lowers the maximum soil temperature, raises the minimum soil temperature and retains moisture, resulting in a high emergence count in soybean (Sarmah, 1986; Chen and Shui, 1996). Not only the emergence but also the grain yield of soybean is improved by straw mulching (Liu, 1996; Lal, 1998). The sowing period of soybean in India coincides with the monsoon rains. Raindrop impact causes the physical compaction of the soil surface and eventually the formation of a crust. Soil strength increases after wetting and subsequent drying, and is greatest in soils with high clay content and large amounts of rainfall (Lee et al., 1996) and under high solar heat conditions (Drew and Hughs, 1974). Seedling emergence has been reported to decrease following soil crust formation (Agrawal and Sharma, 1980; Lee et al., 1996). Straw mulch decreases the crust strength by reducing the force of raindrop impact (Ranganatha and Satyanarayana, 1979), thus ensuring much better emergence than in non-mulched plots (Agrawal and Sharma, 1980). Similarly, the application of FYM as mulch has been reported to improve emergence (Agrawal and Sharma, 1980).

The imbibition of water by seeds is a pre-requisite for germination and emergence. This process could be hastened by soaking the seeds in water for a certain period before sowing. This is also known as ‘on-farm seed priming’ (Harris et al., 2001; Rashid et al., 2004). On-farm seed priming ensures better and quicker emergence than the use of non-primed seed. A seed priming period of 6–8 h for mungbean (Rashid et al., 2004), 8 h for chickpea (Harris et al., 1999), 8 h for wheat (Harris et al., 2001) and 24 h for maize (Harris et al., 1999) has been found optimum. Primed seeds result in a crop which is healthy and matures early, thus escaping pest damage and providing higher yields.

There was thus a need to study the effect of wheat straw mulch, farmyard manure mulch and seed priming on the emergence of soybean. Further, as the sowing period of soybean coincides with the monsoon rains, which result in crust formation and reduce emergence, the effect of different treatments on alleviating the crust effect on the emergence of soybean was also studied.

Materials and methods

Three field experiments were conducted at the Punjab Agricultural University, Ludhiana, India to study the effect of wheat straw and FYM mulches, seed priming and soil crust breaking on the emergence of soybean. The emergence count was studied under normal conditions in Experiments 1 and 2 and under crusted soil conditions in Experiment 3. The soil of the experimental site was loamy sand. A basal dose of 30 kg N and 60 kg P_2O_5 ha^{-1} was applied. Data on the maximum temperatures experienced by the crop are presented in Figure 1.

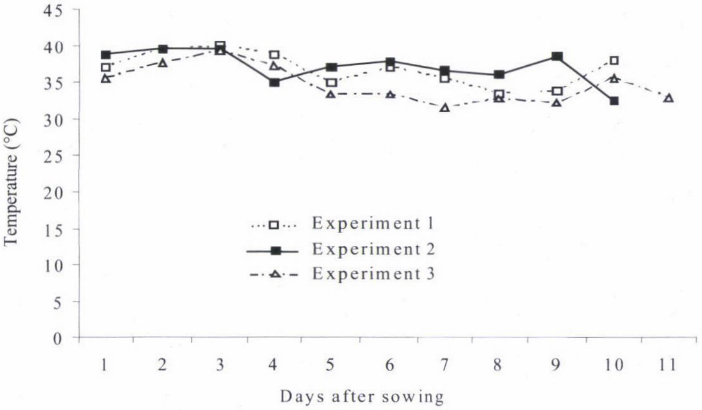

Fig. 1. Daily maximum temperature experienced by the crop in three experiments

Experiment 1

Seven treatments, given in Table 1, were tested in a randomized complete block design having four replications. Each plot measured 5.0 m × 2.25 m. Wheat straw was used at 6 t ha^{-1} to cover the whole plots (plot mulch) or at 3 t ha^{-1} to cover the rows only (row mulch). FYM was used at 10 t ha^{-1} to cover the whole plot (plot mulch) or at 5 t ha^{-1} to cover the rows only (row mulch). Seed priming was done by soaking the seeds in ordinary water for 8 or 16 h. Mulching with wheat straw or FYM was done immediately after sowing, whereas seed priming was done before sowing. Mulch application was done by hand. These treatments were compared with a non-mulched and non-primed treatment. Cultivar SL 295 was sown on 15 June 1999 in rows 45 cm apart using 75 kg seed ha^{-1}. The emergence count was recorded 3, 4, 5, 6 and 7 days after sowing (DAS) from two spots per treatment by counting the number of seedlings emerging per metre row length. Averages were then calculated and the data were converted into emergence count m^{-2}.

Experiment 2

Seven treatments, given in Table 2, were tested in a randomized complete block design having four replications with a plot size of 5.0 m × 2.25 m. Wheat straw was used to cover the whole plot or rows only. In Experiment 1, it was found that plot mulch with FYM was not effective in improving the emergence count, so in this experiment only row mulching was done with 5 t FYM ha^{-1}, either alone or combined with row mulching with 3 t wheat straw ha^{-1}. The mulches were applied by hand. The seed priming period tested was 4 or 8 h in ordinary water. There was a non-mulched non-primed control treatment. Sowing was done on 13 June 2000 using a seed rate of 75 kg ha^{-1} of cultivar SL 295 at 45 cm row spacing. The emergence count was recorded 3, 4, 5, 6, 7 and 8 DAS, as described in Experiment 1.

Experiment 3

In this experiment the emergence was studied under crusted conditions created by simulated rain. The sowing of the experiment, having nine treatments (Table 3), was done on 4 July 2000, in rows 45 cm apart, using a seed rate of 75 kg ha^{-1} of cultivar SL 295. The plot size was 4.0 m × 1.35 m. A few hours after sowing, 1 cm simulated rain was applied by spraying water through a loose nozzle, using a knapsack sprayer. The nozzle was kept loose to enable the formation of bigger droplets and the large quantity of water that could be expected through rainfall. Simulated rain and high temperature resulted in the formation of a crust.

Wheat straw mulch was applied by hand either before or after the simulated rain, as per the treatment, at 6 t or 3 t ha^{-1} to cover the whole plot or the rows only, respectively. Similarly, as per the treatment, FYM at 5 t ha^{-1} was applied by hand either before or after the simulated rain to cover rows only. In the crust breaking treatments, the crust was broken either after 2 days or 4 days using a small hand tool, taking care not to damage the emerging seedlings. Data on the emergence count were recorded 3, 4, 6, 7, 8, 9 and 10 DAS, as reported in Experiment 1.

Statistical analysis

All data were subjected to analysis of variance in a randomized complete block design as per standard procedures.

Results

Emergence under normal conditions

Wheat straw mulch showed a profound effect on improving the emergence count (Tables 1 and 2). It also enhanced the speed of emergence. Initially the emergence count in row mulching with 3 t wheat straw ha^{-1} was low as compared to plot mulching with 6 t wheat straw ha^{-1} during both the years. However, subsequently both treatments were on par in Experiment 1, whereas plot mulching maintained its edge over row mulching till 8 DAS in Experiment 2.

Plot mulching with 10 t FYM ha^{-1} and row mulching with 5 t FYM ha^{-1} were on par for emergence (Table 1), but both were inferior to plot and row mulching with straw. Row straw mulching integrated with row FYM mulching improved the emergence count over row straw mulching alone (Table 2) and was as effective as plot straw mulching.

Seed soaking in water for 8 or 16 h (Table 1) or for 4 or 8 h (Table 2) failed to improve emergence; in fact, emergence was reduced considerably. The adverse effect of seed soaking increased as the period of soaking increased.

Overall, the emergence count in the various treatments was lower in Experiment 2 (Table 2) than in Experiment 1 (Table 1), possibly due to variations in the climatic conditions in the two years of the study. The maximum temperature during the emergence period was higher in Experiment 2 than in Experiment 1 (Fig. 1), resulting in a greater loss of moisture due to evaporation and thus surface drying, which ultimately led to a poor emergence count.

Emergence under crusted soil conditions

Wheat straw mulch, used to cover either the whole plot or rows only, showed a dramatic effect on improving both the total emergence count and the speed of emergence (Table 3). Straw mulch application before the simulated rain had a slight edge over its application after the rain. FYM mulch application and mechanical crust breaking were also effective in improving emergence compared with the control (crusted) treatment.

Table 1

Emergence count of soybean as influenced by wheat straw mulch, FYM mulch and seed soaking under normal conditions (Experiment 1)

Treatment	Emergence count (No. of seedlings m^{-2})				
	Days after sowing				
	3	4	5	6	7
Plot mulch with 6 t wheat straw ha^{-1}	25.1	39.1	45.1	45.1	45.7
Row mulch with 3 t wheat straw ha^{-1}	15.5	33.3	45.1	48.0	48.0
Plot mulch with 10 t FYM ha^{-1}	1.3	12.4	29.5	35.5	37.7
Row mulch with 5 t FYM ha^{-1}	2.8	17.7	28.8	36.2	36.8
Seed soaking in water for 8 h	0.0	8.0	12.4	15.5	15.5
Seed soaking in water for 16 h	0.0	3.5	7.3	9.5	10.2
Control (normal sowing)	0.6	6.6	22.8	28.8	29.5
C.D. 5%	7.2	7.6	8.5	9.5	8.7

Table 2

Emergence count of soybean as influenced by wheat straw mulch, FYM mulch and seed soaking under normal conditions (Experiment 2)

Treatment	Emergence count (No. of seedlings m^{-2})					
	Days after sowing					
	3	4	5	6	7	8
Plot mulch with 6 t wheat straw ha^{-1}	12.9	25.9	37.0	40.0	41.4	42.9
Row mulch with 3 t wheat straw ha^{-1}	2.2	11.8	20.3	25.9	27.7	35.1
Row mulch with 5 t FYM ha^{-1}	0.0	3.7	9.0	14.4	16.6	20.3
Row mulch with 5 t FYM ha^{-1} + 3 t wheat straw ha^{-1}	3.3	24.4	31.1	35.1	37.0	42.2
Seed soaking in water for 4 h	0.0	2.9	6.6	9.2	11.1	12.5
Seed soaking in water for 8 h	0.0	0.7	1.8	2.2	5.1	5.9
Control (normal sowing)	0.0	4.8	10.0	15.9	21.8	24.4
C.D. 5%	4.3	5.0	8.9	9.6	8.8	8.5

Table 3

Emergence count of soybean as influenced by various practices under crusted conditions created by simulated rain (Experiment 3)

Treatment	Emergence count (No. of seedlings m^{-2})						
	Days after sowing						
	3	4	6	7	8	9	10
Rain; crust formed (control)	1.2	7.5	18.1	22.5	27.5	28.8	28.8
Rain; crust broken after 2 days	1.2	9.2	30.7	31.4	35.0	39.0	39.0
Rain; crust broken after 4 days	2.5	16.1	28.1	31.1	38.7	41.8	42.9
Plot mulch with 6 t wheat straw ha^{-1}; rain	13.5	33.8	49.0	51.6	52.4	53.5	53.5
Row mulch with 3 t wheat straw ha^{-1}; rain	9.0	26.8	42.7	46.2	47.9	51.4	51.4
Row mulch with 5 t FYM ha^{-1}; rain	3.7	19.4	31.2	36.1	40.7	44.0	44.0
Rain; plot mulch with 6 t wheat straw ha^{-1}	2.0	16.6	47.4	48.1	50.1	50.5	50.5
Rain; row mulch with 3 t wheat straw ha^{-1}	2.5	15.1	34.4	38.8	42.5	47.4	47.4
Rain; row mulch with 5 t FYM ha^{-1}	0.7	10.9	24.6	27.7	36.4	37.7	37.7
C.D. 5%	5.1	7.3	12.4	11.2	13.3	11.4	11.9

Discussion

For both plot and row mulching, wheat straw mulch resulted in a higher emergence count than FYM mulch (Tables 1 and 2). In various summer legumes, average seedling emergence was reported to be higher with 5 t wheat straw mulch ha^{-1} (67.49%) than with 5 t FYM ha^{-1} (54.5%) (Chaudhari and Das, 1980). Better coverage of the soil surface by wheat straw than by FYM might have resulted in lower temperature and higher soil moisture content and ultimately in a higher emergence count.

Under crusted conditions, the application of straw mulch to either the plot or the rows improved the emergence of soybean when applied both before and after simulated rain (Table 3). The application of FYM mulch before simulated rain and the breaking of the crust also improved emergence (Table 3). Singh (1979) studied the effect of simulated rainfall on the seedling emergence of cotton and pearl millet and reported that seedling emergence under various management practices was in the order: wheat straw applied before rain > rice husk applied over the row after rain > FYM mixed in the surface soil before rain > mechanical breaking of the crust > control (crusted). In the present study the effects of wheat straw mulch, FYM mulch and crust breaking were similar. Wheat chaff and FYM applied in strips over the rows have been reported to increase the percentage and rate of emergence of pearl millet under surface crust conditions (Agrawal and Sharma, 1980).

Plot and row mulching with wheat straw were on par for emergence count in Experiment 1 (Table 1), but in Experiment 2, plot mulching with straw had an edge over row mulching (Table 2), though both had a marked effect compared with the control plot. According to Sekhon and Kaul (1978) wheat straw mulching at 4 t ha^{-1} increased the plant stand of soybean by 104 and 269% compared to the non-mulched control in two years of investigation. This clearly shows the profound effect of straw mulch on the plant stand, as well as the year to year variability in the results, as was also observed in the present studies. This could be due to a variety of reasons, including variation in weather conditions, soil type, soil moisture at sowing, etc. During the observation period, the maximum temperature was higher in Experiment 2 than in Experiment 1 (Fig. 1), which could have resulted in greater loss of moisture due to evaporation and thereby faster surface drying, which might have led to the poor emergence of soybean seedlings.

Straw mulch provides many advantages. It may improve not only emergence but also the growth and grain yield of soybean. Rice straw mulch was reported to increase the grain yield of soybean from 0.95 to 1.25 t ha^{-1} (Adisarwanto, 1985). The nodulation of soybean was improved by wheat straw mulching at 4 t ha^{-1} (Sekhon and Kaul, 1978). Better nodulation and consequently higher nitrogen fixation may result in better plant growth and high grain yields of soybean. Furthermore, higher nitrogen fixation could not only help to meet most of the nitrogen requirement of the soybean crop itself, but also a considerable amount of nitrogen could be left after its harvest for utilization by the succeeding crop in the cropping system. This practice, therefore, could help to reduce the production costs of the cropping system, resulting in higher profits. Furthermore, straw mulch decreases the build-up of salt in the soil (Liu, 1996). Therefore, in salt-affected soils the use of straw mulch may increase the emergence count and grain yield of soybean not only by lowering soil temperature and increasing soil moisture content, but also by reducing the salt content. Straw mulching not only promotes the rapid, uniform germination of soybean seeds, but also offers good weed control and reduces evaporation from the soil surface (Kimura, 1989). These associated advantages of straw mulching have profound benefits in agriculture. Reduced evaporation from the soil surface is not only important for obtaining high grain yields of soybean under rainfed conditions but also under irrigated conditions, as this practice has the potential to reduce irrigation requirements and thereby help save underground water for future generations. This practice is especially important for those areas (such as Punjab and Haryana in India) where the underground water level is dropping at an alarming rate. Soil moisture retention and porosity increased, bulk density decreased and aggregation improved when the quantity of barley straw mulch was increased from 0 to 4 t ha^{-1} (Kim et al., 1991). However, a high mulch load may reduce the plant population in soybean, so the mulch quantity must be carefully chosen to avoid an adverse effect on the emergence count.

Not only wheat straw (Tables 1, 2 and 3; Sarmah, 1986) but also paddy straw (Sarmah, 1986; Chen and Shui, 1996) has been found to improve the emergence of soybean. Both are available in large quantities, but paddy straw is generally burnt in the field, as it has fewer alternative uses. This straw could be utilized for improving the emergence of soybean under both normal and crusted soil conditions. Rainfall before emergence results in a very poor plant stand, thus demanding re-sowing, which the farmers cannot afford due to the very high cost of seed. The use of mulches to obtain good emergence and ultimately high grain yields could encourage farmers to grow soybean on a large acreage. In areas where straw mulches are not available FYM mulch could be used or the crust could be broken mechanically.

Breaking the crust improved the emergence count of soybean (Table 3) and has been reported to significantly improve cotton emergence (Montemayor, 1998). Crusts should be broken with care, otherwise the emerging seedlings may be damaged. Furthermore, improved implements need to be developed for breaking soil crusts, as reported for cotton (Montemayor, 1998).

The fact that seed priming of soybean in water reduced emergence rather than improving it could possibly be due to injury during water uptake. Other authors reported that the emergence of soybean accelerated by 2–3 days when the seed was soaked in water for as long as 24 or 48 h prior to sowing (Uslu et al., 1997), so different genotypes of soybean may have a differential germination response to seed soaking. Wadud and Kosar (1997) reported that soybean genotypes from Peshawar (Pakistan) produced the highest germination percentage without soaking and that soaking these genotypes for more than two hours prevented germination completely. Similarly, Chachalis and Smith (2000) also reported reduced germination in some grain legumes after seed soaking. In the case of mungbean, seeds soaked for 8 h emerged earlier and with a higher number of emerged seedlings compared to non-soaked seeds (Arif et al., 2003). Rashid et al. (2004) also reported that for mungbean the optimum soaking time was 6–8 h and that soaking for 12 h reduced the grain yield. However, in the present study on soybean, seed priming for 4–16 h reduced emergence.

Even short periods under water may cause water uptake injury to imbibing soybean seeds, but this injury can be avoided by osmotically reducing the initial rate of water uptake. It was shown that soaking in 30% polyethylene glycol (PEG) solution did not injure the seeds (Woodstock and Taylorson, 1981), while seed treatment with 40% PEG solution considerably improved the germination of soybean over the untreated control (Meng and Li, 1992). This shows that seed soaking should be done not in ordinary water but in PEG solution to avoid any injury to the imbibing seeds due to fast water uptake.

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EFFECT OF ORGANICS ON NITROGEN TRANSFORMATIONS IN SOIL UNDER DIFFERENT MOISTURE REGIMES

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Awareness of the environmental aspects of the quality of crop production has increased in recent decades, leading to renewed interest in organics such as crop residues, green manures and organic manures. The effect of organics on urea transformation was investigated by conducting a laboratory incubation experiment in alluvial clay loam soil (Typic Ustifluvents) at 33±1°C with two moisture levels (1:1 soil:water ratio and field capacity). The rate of urea hydrolysis decreased as the time of incubation increased and the disappearance of urea N was associated with a corresponding increase in the (NH_4^+ + NO_3^-)-N content in soils treated with crop residues (rice straw and wheat straw), organic manures (poultry manure and farmyard manure) and green manures (cowpea and sesbania). In untreated soil, the time taken for the complete hydrolysis of the applied urea (200 μg urea N g^{-1} soil) was more than 96 h at both the moisture levels, whereas in amended soils it was completed in 48 h. The rate of urea hydrolysis was more rapid at field capacity than at the 1:1 soil:water ratio. Urea hydrolysis was higher in sesbania-treated soils, followed by cowpea, poultry manure, farmyard manure, rice straw and wheat straw at both the moisture levels. At field capacity, 85.5% urea was hydrolysed in sesbania-treated soil as compared to 32% in untreated soil after 24 hours of incubation, while at the 1:1 soil:water ratio the corresponding values were 81.5 and 27.5%. Urea hydrolysis followed first order reaction kinetics at both the moisture levels.

Key words: urea transformation, urea-N, organics, kinetics, urea hydrolysis, moisture regimes

Introduction

In recent years, fertilizer costs and concern for sustainable soil productivity and ecological stability in relation to chemical fertilizer use have emerged as important issues (Aulakh and Singh, 1997). There is renewed interest in the integrated use of mineral fertilizers with organics such as farmyard manure, green manures and crop residues as a source of plant nutrients

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(Ghosh et al., 2004). The addition of organic material to soil results in increased organic matter, crop productivity and soil biological activity (Collins et al., 1992).

Urea fulfils 80% of the total N demand in India. However, the efficiency of urea is low (30–50%) for all crops due to various N loss mechanisms. Urea transformation involves the initial cleavage of the urea molecule into ammonia and carbamic acid by the enzyme urease and subsequent chemical hydrolysis into ammonia and carbon dioxide. Urea hydrolysis is maximum in soils with high organic C, pH around 8.0, moisture at field capacity and temperature around 35°C (Panda, 2005). Khind et al. (1991) reported that urea hydrolysis occurs more rapidly in soil treated with sesbania, and increases with a higher concentration of organic amendment and with a longer period of incubation. Urea hydrolysis rates can be changed quickly by the addition of crop residues up to 7 days of decomposition (Saini et al., 1994). The efficient management of N is possible when a clear understanding of nitrogen transformation from N sources is developed. However, meagre information is available on the effect of the addition of crop residues and organic manures on the transformation of urea. Therefore, a laboratory incubation study was undertaken to investigate the effect of crop residues, organic manures and green manures on urea transformation in Typic Ustifluvents at two moisture regimes.

Materials and methods

The study was a laboratory incubation experiment conducted to investigate the effect of organics such as green manures, crop residues and organic manures on urea transformation in the soil. The crop residues included rice straw (RS) and wheat straw (WS), the organic manures were poultry manure (PM) and farmyard manure (FYM) and the green manures were cowpea (CP) and sesbania (S). A bulk surface (0–15 cm) soil sample was collected from the Ludhiana district (30°50' N and 75°41' E) of Punjab, India (Typic Ustifluvents-alluvial clay loam) and the processed (<2 mm) soil sample was analysed for various physico-chemical characteristics. Soil texture was determined using the International Pipette Method (Day, 1965) and the organic carbon by the method given by Walkley and Black (1934). The soil contents of alkaline $KMnO_4$-oxidisable N (Subbiah and Asija, 1956), 0.5 M $NaHCO_3$ (pH 8.5)-extractable P (Watanabe and Olsen, 1965) and 1 N NH_4OAc (pH 7.0)-extractable K (Pratt, 1982) are given in Table 1.

From the processed soil sample, 5 g portions were placed in a series of plastic containers. Finely ground crop residues and organic manures were mixed @ 2% (w/w) with the soil and 1 ml distilled water was added to each container to bring the soil to field capacity. A control treatment without crop residues and organic manures was also prepared. The soil samples were incubated for 15 days to decompose the organic material. This study was conducted at two moisture levels (field capacity and 1:1 soil:water ratio). Urea was applied on the surface of the soil in solution form @ 200 μg urea N g^{-1} soil. After treating the soil with urea, the plastic containers were incubated at 33±1°C for periods of 12, 24, 36, 48, 60, 72 and 96 hours. The soil samples (5 g) were immediately extracted (1:10 soil:extractant ratio) with 2 M KCl containing 5 μg ml^{-1} of phenyl mercuric acetate (PMA) and the extracts were analysed for urea N by the diacetylmonoxime method (Mulvaney and Bremner, 1979) and for (NH_4^+ + NO_3^-)-N by the distillation method (Bremner and Keeney, 1966) using magnesium oxide and Devarda's alloy.

Table 1

Physico-chemical characteristics of the experimental soil

Sand (%)	27.5
Silt (%)	38.3
Clay (%)	34.2
Texture	Clay loam (Typic Ustifluvents)
pH (1:2 soil:water suspension)	7.62
EC (dS m^{-1})	0.63
OC (%)	0.35
Alkaline $KMnO_4$-N (mg kg^{-1})	62.9
0.5 M $NaHCO_3$- extractable P (mg kg^{-1})	5.7
1 N NH_4OAc-extractable K (mg kg^{-1})	200.8
$(NH_4^+ + NO_3^-)$-N (mg kg^{-1})	42

Kinetics of urea transformation

The following zero- and first-order rate kinetic models were used to describe the urea transformation:

$$C_t = C_o + K_t \text{ (zero-order)}$$

$$\ln C_t = \ln C_o - K_t \text{ (first-order)}$$

where K – specific first order rate constant (h^{-1}); C_o – initial concentration of added urea N (μg); C_t – concentration of urea N in soil solution at time 't'; t – time interval (h).

Results and discussion

The effect of crop residues (rice straw and wheat straw), organic manures (farmyard manure and poultry manure) and green manures (cowpea and sesbania) on the transformation of applied urea (200 μg urea N g^{-1} soil) at different incubation intervals and soil moisture regimes is shown in Figures 1 and 2. The time taken for the completion of urea hydrolysis was more than 96 h in untreated soils, whereas in amended soils it was completed within 48 h at both the moisture levels (1:1 soil:water ratio and field capacity). The high rate of urea hydrolysis at field capacity might be due to the greater urease activity at field capacity than at the 1:1 soil:water ratio. Agehara and Warneke (2005) also observed increased urease activity at field capacity. The highest rate of urea hydrolysis was observed in sesbania-treated soils, followed by CP, PM, FYM, RS and WS after all the incubation periods at both moisture levels. This may be due to the fact that sesbania had the lowest C:N ratio (19:1) and the highest urease activity (75.3 μg urea N hydrolysed g^{-1} soil h^{-1}), as compared to the other amendments. The low urease activity in wheat straw may be due to its wider C:N ratio. This indicates that the addition of crop residues and organic manures stimulates microbial activity, with the subsequent synthesis of urease in the soil. The results are in conformity with the findings of Reddy and Chhonkar (1991) that the urease activity in soil and floodwater increased significantly due to the addition of organic matter, and was related to the C:N ratio of the organic materials.

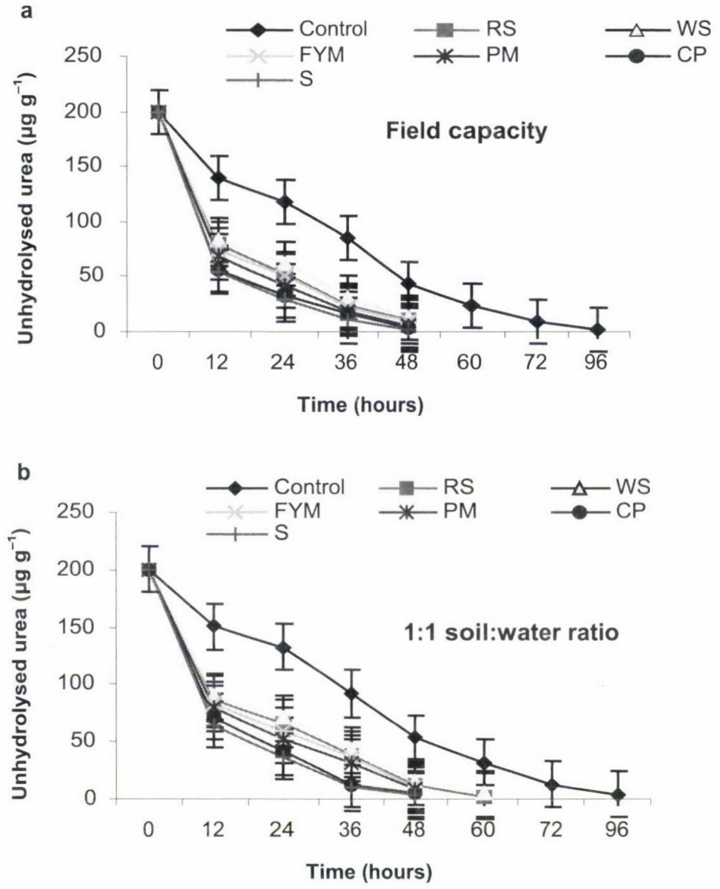

Fig. 1. KCl-extractable urea-N at different periods of incubation in alluvial clay loam soil at field capacity and 1:1 soil:water ratio. Vertical bars indicate standard errors. RS: rice straw; WS: wheat straw; FYM: farmyard manure; PM: poultry manure; CP: cowpea; S: Sesbania

The decrease in urea N was paralleled by a concomitant increase in mineral N ($NH_4^+ + NO_3^-$) concentrations with an increased period of incubation in both amended and unamended soils at both moisture levels. In treated soils with a 1:1 soil:water ratio, the amount of (NH_4^++NO_3^-)-N was 125, 117, 143, 156, 170 and 186 µg g^{-1} soil after 24 h of incubation in the RS, WS, FYM, PM, CP and S treatments, respectively, which increased after 48 h of incubation. Similarly, in the case of field capacity, the amount of (NH_4^++NO_3^-)-N was 145, 131, 153, 164, 173 and 185 µg g^{-1} soil in the respective treatments, which also increased after 48 hours of incubation. Consequently, it was highest in sesbania-amended soil for both the moisture regimes. Aulakh et al. (2001) also found increased rates of urea transformation following the application of organic matter and crop residues, which may have long-term benefits for the yields of crops. A peak concentration of mineral N occurred after 48 hours of incubation at field capacity, whereas it occurred after 60 hours of incubation at the 1:1 soil:water ratio (Fig. 1a, b). This may be due to the higher degree of urea hydrolysis at field capacity. The results are consistent with the observations of Wali et al. (2003) that the rate of urea hydrolysis was faster at field capacity than under water-logged conditions due to the depletion of oxygen, which is a prerequisite for microbial activity, under water-logged conditions.

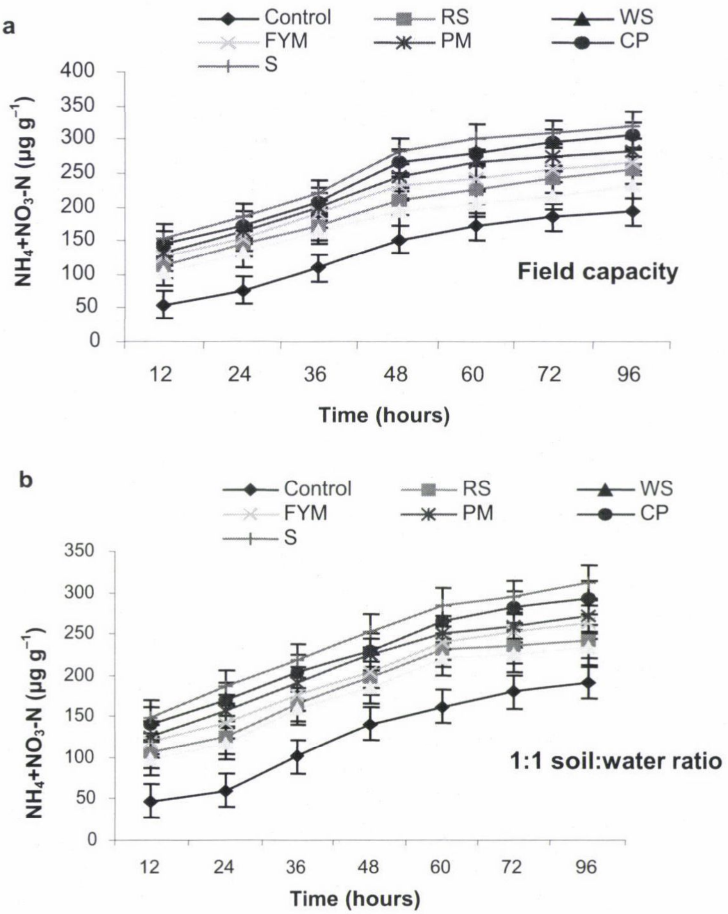

Fig. 2. KCl-extractable (NH_4+NO_3)-N at different periods of incubation in alluvial clay loam soil at field capacity and 1:1 soil:water ratio. Vertical bars indicate standard errors. RS: rice straw; WS: wheat straw; FYM: farmyard manure; PM: poultry manure; CP: cowpea; S: Sesbania

Soil amended with green manure such as sesbania hydrolysed significantly higher quantities of urea at a faster rate than organic manures (PM and FYM) or crop residues (RS and WS). In the sesbania-amended soil, 86% urea was hydrolysed at field capacity (Fig. 2a) as compared with 80 and 74% in PM and RS-amended soil after 24 hours of incubation, while the respective values were 81, 74 and 67% for the 1:1 soil:water ratio. This may again be due to the wider C:N ratio of organic manures and crop residues, which decompose at a slower rate than green manures.

In the present study, a first-order equation gave the best description of the urea transformation with organic manures, crop residues and green manures, as evidenced by the higher value of the coefficient of determination (R^2; Table 2). The plot of ln ($C_t - C_o$), i.e. the logarithm of the concentration of unhydrolysed urea against time 't', indicated that urea hydrolysis followed a first-order reaction in all the treatments under both moisture regimes. Sankhayan and Shukla (1976) and Singh (1990) also reported that urea hydrolysis followed a first-order reaction. In unamended soil, the rate constant (K) had a value of 0.067 at 1:1 soil:water ratio and 0.069 at field capacity. In amended soils, the value of the rate constant ranged from 0.090 to 0.116 and from 0.102 to 0.121 at the 1:1 soil:water ratio and at field capacity, respectively. The average time for 50% (t½) hydrolysis in unamended soils was 10.01 hours at the 1:1 soil:water ratio and 10.20 hours at field capacity. In the amended soils, the values of t½ ranged from 6.48 to 7.64 hours at the 1:1 soil:water ratio and from 6.50 to 6.95 hours at field capacity (Table 3).

Table 2

Values of the coefficient of determination (R^2) of the kinetic models in alluvial clay loam

Treatment	Zero-order		First-order	
	1:1 Soil:water	Field capacity	1:1 Soil:water	Field capacity
Control	0.80	0.77	0.91	0.93
Rice straw	0.84	0.89	0.93	0.94
Wheat straw	0.88	0.91	0.96	0.92
Farmyard manure	0.92	0.88	0.93	0.95
Poultry manure	0.91	0.87	0.94	0.97
Cowpea	0.84	0.86	0.98	0.98
Sesbania	0.82	0.82	0.99	0.95

Table 3

Values of the rate constant (K) of the first-order equation for different treatments in alluvial clay loam

Treatment	Moisture level			
	1:1 Soil:water		Field capacity	
	K	t 1/2	K	t 1/2
Control	0.067	10.2	0.069	10.01
Rice straw	0.095	7.23	0.104	6.73
Wheat straw	0.090	7.64	0.102	6.63
Farmyard manure	0.096	6.48	0.108	6.76
Poultry manure	0.105	6.53	0.111	6.80
Cowpea	0.112	6.80	0.117	6.95
Sesbania	0.116	6.76	0.121	6.50

The value of the rate constant was low in sesbania-treated soils and highest in soil amended with wheat straw. These values indicate that the transformation of urea varies with the C:N ratio of the applied organic materials. Boggs et al. (2000) also reported that the decay rate is inversely related with the C:N ratio of the applied organic material. Therefore, the higher the C:N ratio, the lower the urease activity, which resulted in the lower hydrolysis of urea. The differential rate constant and t½ values in amended soils could perhaps be attributed to the higher decomposition activity of the urease enzyme (Frankenberger et al., 1983). Of all the amended soils, sesbania had the highest rate constant and lowest t½ values due to having the lowest C:N ratio and higher urease activity. Vigil and Kissel (1991) and Parr and Papenduck (1978) also observed that crop residues with a wide C:N ratio decompose more slowly than those with a narrow C:N ratio. Further, the values of the rate constant were higher at field capacity than for the 1:1 soil:water ratio, which may be ascribed to the increased urease activity at field capacity compared with the 1:1 soil:water ratio. Saharawat (1980) also reported increased urease activity with an increase in the moisture content up to field capacity, after which it remained constant with a further increase in moisture content.

The C:N ratio was significantly negatively correlated with urease activity (r = –0.951*). It is clear that organic amendments having a higher C:N ratio decompose at a slower rate, thereby lowering the availability of nitrogen. Singh and Bajwa (1986) also reported a negative correlation between urease activity and the C:N ratio. The urease activity was positively correlated with the rate constants at both the moisture levels (Table 4). However, the relatively higher value of the correlation at field capacity (r = 0.990) confirms the higher amount of mineral N at this moisture level than at a 1:1 soil:water ratio.

Table 4

Correlation between urease activity, C:N ratio and rate constants

	Urease activity	C:N ratio
C:N ratio	–0.951*	–
Rate constant at field capacity	0.990*	–0.898*
Rate constant at 1:1 soil:water ratio	0.976*	–0.922*

* Significant at the $P \leq 0.05$ level

Conclusions

The results of the present study indicated that urea hydrolysis proceeded more rapidly at field capacity than at a 1:1 soil:water ratio due to higher urease activity. The rate of urea hydrolysis was the highest in sesbania-treated soils, followed by cowpea, poultry manure, farmyard manure, rice straw and wheat straw under both the moisture regimes. Urea hydrolysis followed first-order reaction kinetics in all the treatments under both moisture regimes. Information on integrating fertilizer N with organics or their use as an alternative source of nutrients will contribute greatly to the development of sustainable agricultural management systems.

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RESIDUAL EFFECT OF LEGUMINOUS CROPS AND INORGANIC FERTILIZER ON SOIL PROPERTIES AND MAIZE GRAIN YIELD

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Soil management practices that utilize organic matter have great potential to increase productivity in sub-Saharan Africa. Field studies were carried out between September 1995 and August 1998 to determine the effects of three leguminous crop species: velvet bean (*Mucuna pruriens* var. *utilis*), groundnut (*Arachis hypogaea* L.) and cowpea (*Vigna unguiculata* (L.) Walp), and inorganic fertilizer on the soil properties and succeeding maize grain yield when grown in rotation on a sandy soil classified as Haplic Lixisol in the forest-savannah transition zone of Ghana. The legumes were established in the minor seasons and maize in all the plots in the major cropping seasons. A 2 × 3 factorial design laid out in a randomized complete block was used. The main plots consisted of three leguminous crop residues and the sub-plots of two fertilizer levels (0 and 45 kg N ha^{-1}, 19 kg P ha^{-1}, 19 kg K ha^{-1}). The control consisted of maize following maize with the recommended fertilizer rate (90 kg N ha^{-1}, 37 kg P ha^{-1}, 37 kg K ha^{-1}). On average the *Mucuna* plots added 4.0 t ha^{-1} of crop residue to the soil in a season and cowpea 1.0 t ha^{-1}. The preceding crops had little effect on the soil properties. Leaf area index, total dry matter and maize grain yields were significantly affected by fertilizer. The best maize grain yield (6787 kg ha^{-1}) was recorded in the first year on *Mucuna* plots with half the recommended rate of fertilizer. The cropping sequence with *Mucuna* residue was the most efficient. The gap in maize grain yield between the fertilized and unfertilized treatments widened each successive year. The interaction between organic matter and fertilizer may have been limited due to the surface application of the organic residue.

Key words: legume, crop residue, maize, fertilizer and crop rotation

Introduction

The use of fertilizers by small-scale farmers in Ghana to overcome nutrient deficiency is limited by socio-economic constraints. According to Bumb et al. (1994), during the 1970s Ghana experienced an annual increase in fertilizer use of approximately 20%, whereas during the 1980s there was an annual decline of 7.6% due to the removal of subsidies on agrochemicals. Organic resources may provide several benefits compared with mineral fertilizers; however, it is difficult to predict the time of nutrient release and the crop demand. In contrast, mineral fertilizers offer greater flexibility in the time and placement with which they may be applied. Combining organic and inorganic nutrient sources can provide the most efficient use of scarce resources. Encouraging the use of biologically fixed N from leguminous crop species may present an alternative option which, when managed properly, could cut down the fertilizer requirement of farmers considerably. Grain legume–cereal rotations provide less organic matter and N in their residue because N is removed with the grain, but they make economic sense to farmers due to the combination of food value and good market value.

The residual benefit from legumes to succeeding crops is often greater than expected, reflecting the fact that nutrients from both above and below ground may be significant. Groundnut and cowpea are the two most important grain legumes in Ghana based on production and consumption, while *Mucuna* is a minor grain legume used as food (Osei-Bonsu et al., 1996). Cowpea is reported to fix 201 kg N ha^{-1} in a season (Singh and Rachie, 1985), groundnut between 50 and 210 kg N ha^{-1} (Bell et al., 1994; Giller, 2001) and *Mucuna* 90 kg N ha^{-1} (Enin et al., 2004). The organic residue of these legumes may also improve nutrient availability to crops by altering soil physico-chemical properties such as pH, microbial biomass, factors influencing soil-P sorption and soil moisture storage capacity, and improving the time of nutrient release to coincide with plant demand. Several studies have focused on the N content of the crop residue in selecting plant material, but the N content alone may not be sufficient to predict the release pattern. The total amount of crop residue, the C:N ratio, the soluble portion of C, the P content of the material and soil pH may influence the release and availability of plant nutrients from the crop residue. If small-scale farmers are to achieve a significant improvement in productivity, there is the need to develop and transfer superior crop varieties and management practices that will conserve and utilize ample rainfall to increase maize productivity and production in a sustainable and environmentally friendly manner. The objective of the study was therefore to determine the effect of the three leguminous crop species and inorganic fertilizer on the soil chemical properties and the succeeding maize grain yield.

Materials and methods

The experiment was conducted at the research station of CSIR-Crops Research Institute at Ejura, Ghana (01°22' W; 07°23' N). The soil properties at the start of the experiment are shown in Table 1. The mean annual rainfall is about 1300 mm with bimodal rainfall distribution. In the forest-savannah transition zone, the major rainfall season is from April to August, and the minor rainfall season from September to November. The soil at the experimental site is a Haplic Lixisol. Plots at the site were slashed, and glyphosate was applied at the rate of 900 g a.i. ha^{-1} to the re-growth two weeks after slashing. A randomized complete block design with four replications was used during the minor cropping seasons. Soil samples were collected from 0–15 cm and 15–30 cm in August 1995 and August 1998 and were analysed using standard laboratory procedures. Three leguminous crops: velvet bean (*Mucuna pruriens* var. *utilis*), groundnut (*Arachis hypogaea* var. Kumawu red) and cowpea (*Vigna unguiculata* var. IT8D-1627) and maize were planted. All the seeds were obtained from CSIR-Crops Research Institute, Fumesua and the legumes were planted on August 20, 1995, September 15, 1996 and September 3, 1997. The control treatment was continuous maize (var. Obatanpa) planted in both seasons at the recommended fertilizer rate of 90 kg N ha^{-1}, 37 kg P ha^{-1}, 37 kg K ha^{-1} (GGDP, 1993). Basal compound fertilizer was applied to the maize at the rate of 37 kg N, P and K ha^{-1} one week after planting (WAP), while urea at the rate of 53 kg N ha^{-1} was top-dressed at 5 WAP. However, no fertilizer was applied to the legumes. Weeds were controlled by hand hoeing at 3 and 6 WAP. Cowpea was sprayed against pre-flowering insects at 35 days after sowing with Karate (at the rate of 15 g lambda-cyhalothrin per hectare) and against post-flowering insects at 45 and 55 days after planting using 400 g dimethoate per hectare. At maturity, plants from the four central rows were harvested. The pods were weighed after drying in the sun, then threshed and the seed weight determined. The aboveground plant parts were cut at ground level from an area of 1 m^2 at harvest and dried in an oven at 70°C for 48 hours to determine the biomass left on the plot after harvesting the pods. The groundnut stover was spread uniformly on the same plots from which they were harvested after picking the pods.

Maize was planted on all the plots in a two-factor randomized complete block design in the major season (April to August). The main plots were three preceding legume stubbles. The second factor consisted of two fertilizer levels (0 and 45 kg N ha^{-1}, 19 kg P ha^{-1}, 19 kg K ha^{-1}) and a control of maize with the recommended rate of fertilizer (90 kg N ha^{-1}, 37 kg P ha^{-1}, 37 kg K ha^{-1}). There were 1.4 m paths between the sub-plots. Maize was planted in all the plots on 20 April, 1996, 8 May, 1997 and 28 April, 1998. Each plot consisted of 9 rows. One week after planting, basal N-P-K compound fertilizer was applied at the rate of 19 kg each of N, P and K ha^{-1} and the plots were top-dressed with urea at the rate of 26 kg N ha^{-1} at 5 WAP. The fertilizer rate for the control plot was 37 kg each of N, P and K ha^{-1} at 1 WAP and 53 kg N ha^{-1} at 5 WAP. At maturity, cobs from three middle rows were harvested without the end hills. The cob weight was determined, and the grain moisture at the time of harvest was measured using a grain moisture meter (Dole moisture meter, Model No. PB-70-21). Soil samples were taken after the maize harvest and analysed as above.

Results

The initial and final soil chemical properties are presented in Tables 1 and 2, respectively. The preceding crops made different contributions to the soil chemical composition. The level of organic carbon increased by 1.5% on the *Mucuna* and groundnut plots, but decreased on the maize and cowpea plots. Total N increased slightly on plots planted to maize, remained constant on the groundnut and velvet bean plots, but decreased on the cowpea plots. The level of P, K, Ca and Mg generally declined in all the plots at the end of three years.

The grain yield and crop residue left on the field by the minor season crops are presented in Table 3. *Mucuna* produced the least grain yield throughout the three years, with a mean grain yield of 369 kg ha^{-1}. Cowpea gave the highest grain yield among the legumes, with a three-year average of 963 kg ha^{-1}, indicating that more nutrients were harvested from the cowpea plots than for the other legumes. However, *Mucuna* left a mean of 4.09 t ha^{-1} of crop residue on the soil during the three years. Cowpea left on average 1.147 t ha^{-1} of crop residue on the soil over the same period. The legume residue did not have a significant effect on the total dry matter (TDM) or leaf area index (LAI). However, the application of 45 kg N ha^{-1}, 19 kg P ha^{-1} and K kg ha^{-1} to maize following the legumes had a significant ($p<0.05$) effect on LAI and TDM (Figs. 1a, 1b, 2a and 2b). At 8 WAP, the average TDM of fertilized maize was 20% more than that of unfertilized maize planted after the legumes in 1996. There was, however, no significant difference in TDM in 1997. The difference in LAI between fertilized and unfertilized plots was significant in both years (19% in 1996; 17% in 1997).

Table 1

Physical and chemical properties of the experimental soil in 1995

Parameter	0–15 cm	15–30 cm
Organic carbon (mg kg^{-1})	6.6	6.5
Total N (mg kg^{-1})	0.42	0.28
P (mg kg^{-1})	12.0	9.0
Exchangeable cations (cmol(+) kg^{-1})		
Ca	2.40	2.30
Mg	1.85	1.17
K	0.20	0.19
Na	0.14	0.13

Table 2

Physical and chemical properties of the experimental soil in August 1998 (0–30 cm)

Previous crop		Organic C (mg kg^{-1})	Total N (mg kg^{-1})	Available P (mg kg^{-1})	Exchangeable cations (cmol (+)kg^{-1}			
					Ca	Mg	K	Na
Mucuna	0–15 cm	6.7	0.42	10.2	1.96	1.02	0.27	0.20
	15–30 cm	6.5	0.32	7.5	1.65	0.63	0.19	0.12
Cowpea	0–15 cm	6.6	0.37	9.8	1.07	1.72	0.25	0.13
	15–30 cm	6.4	0.32	8.1	1.55	0.75	0.21	0.13
Groundnut	0–15 cm	6.7	0.42	9.5	1.75	0.92	0.25	0.15
	15–30 cm	6.5	0.32	8.5	1.60	0.68	0.21	0.14
Maize	0–15 cm	6.6	0.46	10.8	1.89	1.03	0.31	0.16
	15–30 cm	6.5	0.37	8.3	1.61	0.72	0.24	0.13

Table 3

Grain yields and crop residues of the minor season crops (kg ha^{-1})

Crop	Grain yield				Residue			
	1995	1996	1997	Mean	1995	1996	1997	Mean
Mucuna	244	317	369	369	4769	3643	3864	4092
Cowpea	1120	520	1248	963	1339	993	1108	1147
Groundnut	777	657	586	673	2818	2264	2492	2525
Maize	2869	2422	3142	2811	4297	3632	4050	3993

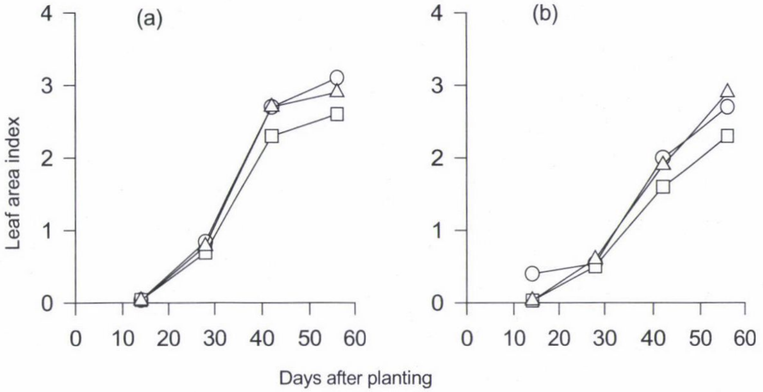

Fig. 1. Effect of preceding crop and fertilizer on leaf area index of maize. a: 1996; b: 1997

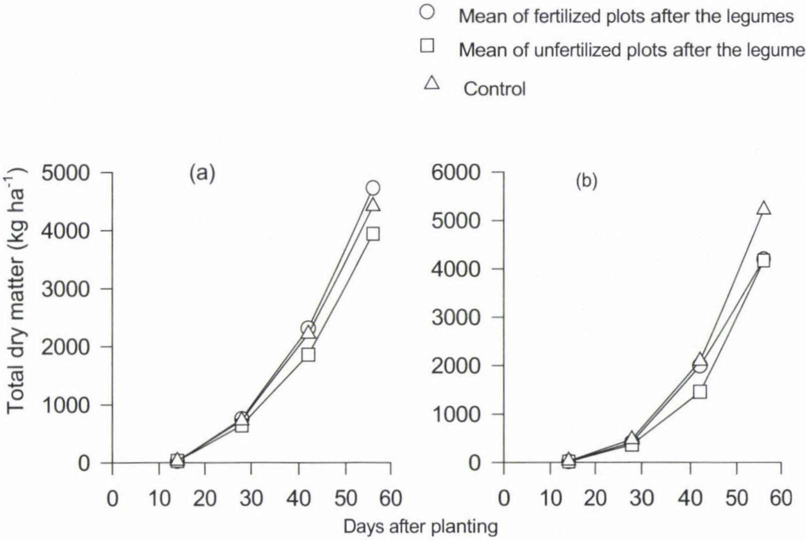

Fig. 2. Effect of preceding crop and fertilizer on total dry matter of maize. a: 1996; b: 1997

The application of half the recommended rate of fertilizer to maize after the three legumes resulted in an increase in grain yield, ranging from 6–13% in 1996, 11–12% in 1997 and 25–36% in 1998 (Table 4). The difference between fertilized and unfertilized plots was less than 14% in the first two years, but during the third year of the experiment the gap between the fertilized and unfertilized treatments widened to 36%.

Table 4

The effects of preceding crop and fertilizer on maize grain yield (kg ha^{-1})

Treatment	1996	1997	1998	Mean
Mucuna + ½NPK	6787	3834	5718	5446
Mucuna without fertilizer	5994	3429	4456	4626
Cowpea + ½NPK	5433	3123	5155	4570
Cowpea without fertilizer	5064	2824	3793	3894
Groundnut + ½NPK	5991	3225	4345	4520
Groundnut without fertilizer	5665	2874	3488	4009
Maize + NPK	6387	3939	5706	5344
Mean	5903	3321	4666	4630
SE	218.9	165.6	335.4	–

NPK = 90–37–37 kg N-P-K ha^{-2}

Discussion

Changes in the soil chemical properties did not follow any consistent pattern. The imposition of the treatments maintained the organic carbon content of the cowpea and maize plots. However, there was a gradual increase in organic carbon in the *Mucuna* and groundnut treatments. The level of P, K, Ca and Mg generally declined in all the plots at the end of three years. The relatively high level of N observed on the control plot may be attributed to the higher amounts of fertilizer applied in the previous seasons. Fertilizer generally led to an appreciable increase in TDM (20%) and LAI (18%), culminating in higher maize grain yields. Planting maize after the leguminous crops without mineral fertilizer resulted in lower yields when compared with the control which received 90 kg N ha^{-1}, 37 kg P ha^{-1} and 37 kg K ha^{-1}. On average, the reduction in maize yield was 718, 1450 and 1335 kg ha^{-1} in the *Mucuna*, cowpea and groundnut treatments, respectively, when grown without supplementary mineral fertilizer for the three-year period. The yield increase attributed to the application of half the recommended rate of fertilizer was 820, 676 and 511 kg ha^{-1} on the *Mucuna*, cowpea and groundnut plots, respectively. In the case of maize after *Mucuna* this yield was 102 kg ha^{-1} higher than in the control treatment, even though the difference was not significant. The leguminous crops may not have contributed sufficient nutrients for optimum grain yield because of the relatively low quality of the crop residue. Nutrient loss through the seed harvest may have led to the low yields recorded in the cowpea and groundnut treatments. Vanlauwe et al. (2000) observed a loss in quality of *Mucuna* residue from 3% at peak biomass to just above 1% at the time of planting the next maize crop. The surface application of crop residues improves the water storage capacity of the soil and reduces erosion and runoff, but does not enhance the interaction between organic matter and fertilizer through the immobilisation of mineral-N, as in the case of incorporation. It may also lead to leaching, as observed by Vanlauwe et al. (2002). The average maize grain yield after

Mucuna without fertilizer (4626 kg ha^{-1}) was not significantly different from that achieved with cowpea or groundnut with half the recommended rate of fertilizer (4570 and 4520 kg ha^{-1}, respectively).

The high maize yield recorded in the *Mucuna* treatment may be attributed to the large biomass produced; the relatively low decomposition rate and the low seed yield may have retained substantial amounts of plant nutrients in the residue. Also, large crop residues may improve the water storage capacity of the soil. Visual observation indicated that cowpea and groundnut residue decomposed faster than *Mucuna*. Cropping systems and practices that improve the water-holding capacity of the soils are not only important in drier regions but also in humid regions, where short-term droughts at critical stages of growth can seriously reduce crop yields. Due to excessive rainfall in 1997, soil nutrients may have been leached, resulting in low maize grain yields. Despite the yield advantage of maize after *Mucuna*, its adoption by farmers will be determined by socio-economic factors and nutritional considerations. The prevailing market prices of maize, cowpea and groundnut will determine which legume to plant in the minor season for higher returns. The study revealed that the application of mineral fertilizer was crucial for maintaining high maize grain yields when organic residues are applied.

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IMPACT OF IMPROVED FALLOW PERIODS ON SOIL PROPERTIES AND PRODUCTIVITY OF MAIZE (*Zea mays* L.) IN MAJOR AND MINOR SEASONS OF ASIAN HUMID TROPICS

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Improved fallows are considered an easy, low cost and suitable method of increasing the productivity and sustainability of smallholder tropical rainfed cropping systems, although most farmers allow weeds to grow when the environmental conditions are not conducive for crop production. Field studies were carried out over the minor and major seasons, to evaluate the impact of a preceding improved fallow using *Crotalaria* or *Tithonia*, two popular tropical green manures, on selected soil properties, and on the growth and yield of maize. Improved fallows enhanced chemical soil properties significantly and the impact was most prominent at the onset of the minor maize season. Thus, the growth and yield of maize was also increased to a greater extent in this season, when yields are generally lower due to the suboptimal climatic conditions of lower rainfall and higher temperatures. However, fallows in the minor season also improved soil characteristics and maize yields in the major season, the most significant impact being increased seed yields and harvest indices. Although farmers may not grow fallow crops in major seasons, the potential of these green manure fallows in increasing maize yields in minor seasons and possible strategies to include the fallows in the cropping sequences of tropical rainfed upland cropping systems are discussed on the basis of this field study.

Key words: improved fallows, soil improvement, maize yields, humid tropics

Introduction

There are now 790 million people in the world lacking adequate access to food, 31% of whom live in South Asia (Pretty et al., 2003). Although modern technologies have increased food production, especially that of rice and maize in the Asian region (Smil, 2000), the challenge today is to enhance productivity on a sustainable basis, without further damaging the environment, which has been increasingly harmed by the haphazard use of agricultural practices, all in the name of increasing productivity (Mc Neely and Scheer, 2001).

A principal feature of environmental damage caused by agriculture is the degradation and nutrient mining of the soil (Hartemink, 2003). In the early 1900s the tropics, especially Asia, were considered a very fertile region. Today, due to fragmentation, intensive cropping and excessive soil mining, a combination of inorganic and organic inputs are advocated to maintain and possibly improve soil fertility and crop productivity in this region (Vanlauwe et al., 2002). Among the many methods of soil improvement, short-term fallows have been identified as a potential agronomic practice for improving soil quality and the fertility of smallholdings in the tropics (Sanchez, 1999). Emphasis has been placed upon the development of more effective and more productive fallows to ensure soil fertility and crop productivity, in contrast to the practice of natural fallows often adopted by smallholders when arable cropping is not feasible for environmental or other reasons.

The use of fallows to increase the productivity of tropical smallholder upland farming systems has been well documented in Africa, where shrub and tree legumes have been used to replace weedy non-productive periods and enhance crop productivity (Ajayi et al., 2003; Whitbread et al., 2004). Emphasis has been placed upon the incorporation of the vegetation grown during the fallow period to improve soil fertility, especially in the short term, as long-term fallows in Africa have not provided the desired benefits when compared to short fallows (Snapp et al., 1998). In contrast to Africa, the emphasis on fallows in Asia has been centred on lowland or upland rice culture (Whitbread et al., 1999; Fagerstrom et al., 2001), where crop residues and *in situ* or *ex situ* vegetation have been incorporated to increase the productivity and sustainability of smallholdings. The use of fallows to increase the productivity of smallholder upland cropping systems in tropical Asia is not as widely documented as for Africa.

Maize is the most important upland cereal in Asia (Devendra and Thomas, 2002; Fuglie, 2004), primarily cultivated as a smallholder crop under rainfed conditions. Yields are generally low in these smallholder systems, and the incorporation of organic matter such as crop residues could increase yields (Sangakkara and Nissanka, 2004). However, the use of improved fallows, especially in the minor seasons when rainfall is generally insufficient for arable cropping, has not been reported in the Asian highland farming context, especially for maize, although studies in Africa report the benefits clearly (Whitbread et al., 2004).

Field studies were undertaken in Sri Lanka over the major and minor seasons that correspond to the two monsoons to evaluate the impact of improved fallows using *Crotalaria juncea*, a popular leguminous green manure, and *Tithonia diversifolia*, a non-legume but common green manure, on the productivity of a rainfed maize crop planted in the following season. The primary objectives of the study were to determine the impact of improved fallows of *Crotalaria* and *Tithonia* over the minor and major seasons on: i) selected soil parameters at the beginning of the maize season after the fallow, and ii) the growth and yields of maize, when compared to the natural fallow used by smallholders especially in the minor dry season, when rainfall could be insufficient for maize.

Materials and methods

The research programme was carried out at the University Experimental Station (418 m above sea level, 8°N, 81°E) of the University of Peradeniya, Sri Lanka, located in the mid-country intermediate zone of the island, over the period June 2003 to August 2004, to encompass the major (wet) and minor (dry) seasons, that correspond to the north east (October – February) and south west (late April – August) monsoons. The soil of the site was an Ultisol (Rhodoult) (Panabokke, 1996), with a sandy clay loam texture. The site receives some 1600 mm of rainfall per annum, with a mean temperature of 31°C.

With the onset of the minor season in late April 2003, 12 plots of dimensions 5 × 5 m were prepared and divided into four blocks. Within each block, which served as replicates, seeds of *Crotalaria* were broadcast or uniform cuttings (5 cm) of *Tithonia diversifolia* were planted at a spacing of 30 × 40 cm on randomly selected plots. The third plot in all blocks was left fallow as per normal practice by farmers when rainfall is insufficient for arable crop production.

At the onset of the rains in the major season (October 2003), the biomass of *Crotalaria*, *Tithonia* and the natural fallow were estimated using 1 × 1 m quadrats and incorporated manually before the plots were prepared for planting. The soil was sampled to a depth of 50 cm (effective root zone) 14 days later for the analysis of selected chemical parameters. Thereafter, maize (*Zea mays*, open pollinated variety Ruwan, germination 91%) was planted at the recommended spacing of 60 × 30 cm (Anonymous, 1989). The fertilizer applied was as per recommendations, and equivalent to 25 kg N, 45 kg P and 30 kg K at planting, followed by 45 kg N at 45 days. Weeding was carried out manually on two occasions.

Soon after establishing the maize (October 2003), another 12 plots of the same dimensions were prepared at an adjacent site and planted with either *Crotalaria* or *Tithonia* or left fallow, as described earlier. At the onset of the rains in the minor season in late April 2004, the biomass of *Crotalaria*, *Tithonia* and natural fallow (weeds) was determined as stated above, and incorporated. The beds were prepared, the soil was sampled at 14 days after incorporation and maize was planted and managed as done in the major season.

In each season, the measurements made on maize were as follows:

Germination (determined on the basis of emergence of the first leaf from the soil at 15 days) within a 1 × 1 m quadrat.

Shoot dry weights (drying at 80°C for 48 hours) of 4 plants per plot taken at 10-day intervals until anthesis for calculating the relative growth rate (RGR) as described by Hunt (1982), and determining the mean RGR over the vegetative growth period.

Leaf area (Li Cor 4000, Li Cor, USA), water potential of the topmost fully opened leaf (Scholander pressure chamber; Scholander et al., 1965) and root dry weights (drying at 80°C for 48 hours) of 4 plants per plot at anthesis.

At crop maturity, number of kernel rows per cob, 1000-kernel weight and seed and stover yields to determine harvest indices.

The soil was analysed 14 days after incorporating biomass for the following:

pH (1:2.5 H_2O), cation exchange capacity (CEC) (ammonium acetate method), soil organic matter (Walkley and Black method), total N (Kjeldhal), P (Olsen, Spectrophotometry) and K (NH_4OAc extraction and flame photometry).

The climatic data were obtained from the records of the Experimental Station and the School of Agriculture, located at a distance of 3 km from the experiment.

The data of the respective seasons and fallow periods were subjected to analysis of variance using a general linear model (GLM). The LSD was used to separate means when the F test was significant (P=0.05), as described by Steel and Torrie (1980), using arc sin transformations when necessary to ensure normal distribution of the data.

Results and discussion

The major season during the north east monsoon was characterized by adequate rainfall, milder temperatures, lower evaporation and higher humidity (Table 1), thus providing a very conducive environment for arable rainfed cropping. The minor season, which corresponds to the south west monsoon, had a significantly lower quantum of rainfall, although the temperatures and evaporation were higher, along with lower humidity. This makes rainfed agriculture difficult on smallholder upland farming systems, where irrigation is not feasible. However, smallholders do cultivate food crops, including maize, in this season, anticipating suitable climatic conditions.

The biomass produced by the improved and natural fallows prior to maize cultivation was significantly greater at the onset of the minor season (Table 2). *Tithonia*, due to its rapid growth and large plant form (Jama et al., 2000), produced 83% and 97% more fresh material for incorporation at the beginning of the minor and major season maize crop, when compared to natural fallow. In contrast, *Crotalaria* produced 57% and 78% more biomass at the beginning of the minor and major maize seasons, respectively, when compared to natural fallow. This suggested a greater capacity of *Tithonia* to withstand the drier conditions than *Crotalaria* in the minor season, making it a more suitable fallow crop. The shorter crop duration of *Crotalaria* in contrast to the perennial *Tithonia* could also hinder its ability to produce a greater quantum of biomass. The natural fallow, consisting of grass weeds (principally *Panicum maximum*) and some broad-leaved species, produced 25% more biomass at the beginning of the minor maize season, although the quantum was the lowest among all the fallow systems in both seasons. Hence, smallholders in tropical Asia could develop an improved fallow system to add better quality organic matter to the soil rather than allow weeds to grow in a natural fallow, as shown under smallholder conditions in the African highlands (Whitbread et al., 2004).

Chemical soil parameters at the onset of the cropping seasons were enhanced by the incorporation of biomass from the improved fallows, in comparison to the natural weedy fallow (Table 3). Soil pH was reduced by the green manures, due to the faster decomposition, thereby releasing organic acids, and the impact was greater at the onset of the minor season. The greater quantum of organic matter added at this time also increased the CEC, with no significant differences between the two green manures. *Tithonia* and *Crotalaria* increased organic matter contents irrespective of the season, and the lack of an interaction between season and fallow indicates that under both environmental conditions, the improved fallow had a greater beneficial impact on soil quality by increasing organic matter than the natural weedy fallow. This phenomenon could enhance crop productivity and sustainability (Snapp et al., 1998).

Table 1

Climatic data of the experimental periods of maize growth

Season	Total rainfall (mm)	Mean temperature (°C)		Mean pan evap. (mm day^{-1})	Humidity (%)
		Air	Soil		
Major – Wet^{+}	1165	28.4 ± 2.1	30.7 ± 1.9	2.65 ± 1.16	78.9 ± 2.9
Minor – Dry^{++}	428	31.4 ± 2.7	32.6 ± 1.6	3.89 ± 0.98	65.7 ± 3.7

evap.: evaporation; $^{+}$October 2003 – February 2004; $^{++}$ April – August 2004

Table 2

Biomass (g m^{-2}) of green manures and weeds added at the end of the major and minor seasons

Season	*Crotalaria*	*Tithonia*	Natural fallow*
Major	562 ± 25.7	654 ± 19.5	357 ± 9.88
Minor	495 ± 16.8	548 ± 11.3	277 ± 7.05

* Weeds consisting of grasses and broadleaved species

Table 3

Soil characteristics as affected by fallow periods in the major and minor seasons
(All measurements taken at the time of planting maize, two weeks after incorporation)

Season	Fallow	pH	CEC	SOM	N	P	K
Major season (Dry fallow)	*Crotalaria*	5.65	39.4	1.46	44.18	12.5	0.65
	Tithonia	5.57	41.5	1.49	38.09	14.6	0.68
	Natural	5.89	36.5	1.38	31.08	11.6	0.58
Minor season (Wet fallow)	*Crotalaria*	5.62	40.5	1.48	51.19	12.8	0.71
	Tithonia	5.57	42.6	1.41	45.10	15.9	0.69
	Natural	5.86	37.2	1.39	33.08	11.9	0.59
LSD(P=0.05)	Fallow	0.04	1.32	0.08	0.05	2.04	0.02
	Season	0.43	0.96	0.21	0.08	3.34	0.04
	Interaction	NS	NS	*	NS	*	NS

pH (1:2.5 H_2O); CEC (m.eq. 100 g^{-1} soil); SOM (%); N (mg g^{-1} soil); P (μg g^{-1} soil); K (m.eq. 100 g^{-1} soil); * Significant at P=0.05; NS: Non-significant

Plant nutrient contents were also increased by the improved fallows (Table 3), again the greatest impact being when the major season was kept fallow, due to the larger quantum of biomass added. As expected, *Tithonia* increased soil P contents due to its mining capacity for this nutrient and the greater concentration in the biomass, irrespective of the season. In contrast, *Crotalaria*, the legume, increased soil N in both seasons. The impact of both improved fallows on the K content was similar and better than the natural fallow. This clearly implies the benefits of maintaining improved fallows for increasing soil properties during seasons when highland smallholder fields in Asia are not cropped, as seen in Africa (Ajayi et al., 2003; Chikowo et al., 2004) and for rice systems in Asia (Fagerstrom et al., 2001).

The use of improved fallow must benefit the succeeding crop to justify inputs and costs (Fagerstrom et al., 2001). The benefits of using the two improved fallows in this study were clearly evident in the vegetative parameters of maize (Table 4). Germination was increased by the improved fallows, especially in the minor season, as the environmental conditions were more conducive for plant growth in the major season. This could reduce the beneficial impact of the green manures. While there were no significant differences between the three fallow systems in the major season, the mean germination of maize seeds was enhanced by 16% in the minor season, due to the incorporation of the improved fallows. This caused a significant saving in seed, a scarce and expensive resource for smallholders who have to purchase seeds for cultivation.

Crotalaria and *Tithonia* fallows increased the relative growth rates (RGR) and leaf area of maize, to a greater extent in the minor season. This again could be attributed to the more conducive environment of the major season, which could reduce the short-term impact of the green manures due to the availability of soil moisture, which is the most limiting environmental factor in the tropics (Turner, 2000). However, the significant interaction between season and fallow in terms of RGR suggests that *Crotalaria*, which has a lower C:N ratio, promotes the vegetative growth of maize to a greater extent in the minor season. In the wet season, the benefits of *Crotalaria* were less evident. The importance of the type of improved fallow was also evident in the leaf area, where the leguminous green manure *Crotalaria* increased leaf expansion to a greater extent than *Tithonia*.

Soil moisture is the most limiting factor in the minor season, as evident from the leaf water potential (Table 4). In the major season, the leaf water potential of maize plants in all treatments was low due to the adequate rainfall. However, improved fallows reduced the values compared with those of plants in a natural fallow, due to the benefits of the green manures on soil moisture retention in tropical Asian soils (Venugopalan and Tarhalkar, 2003). The most significant impact was in the minor season, when the leaf water potential of maize at anthesis declined by 31% due to the use of improved fallows. This clearly highlighted the benefits of improved fallows for increasing the water availability and hence the water content of maize plants in the minor dry season, when crops are subjected to stress conditions.

Root weights were also affected by the fallows (Table 4), and *Tithonia* had a greater influence in promoting the development of the root system, due to the greater P content, confirming earlier studies under controlled conditions (Sangakkara et al., 2004) . Again, greater overall benefits were accrued in the minor season. However, in the major season, even with adequate soil moisture, root dry weights were increased to a greater extent by *Tithonia* than in the dry season, a phenomenon that requires further study.

Table 4
Impact of seasonal fallows on the vegetative growth of maize in major and minor seasons

Season	Fallow	Germination (%)	RGR[a] (mg g^{-1} day^{-1})	Leaf area[b] (cm^{-2})	LWP[b] (MPa)	Root dry wt[b] (g $plant^{-1}$)
Major	*Crotalaria*	88.5	45.8	1569	–3.45	398
	Tithonia	84.7	44.2	1525	–4.21	469
	Natural	82.4	39.5	1436	–5.14	326
Minor	*Crotalaria*	78.3	38.5	1342	–5.95	499
	Tithonia	79.4	33.9	1296	–5.76	518
	Natural	67.8	31.2	1165	–8.06	464
LSD(P=0.05)	Fallow	5.14	2.16	85.47	0.68	20.4
	Season	2.67	1.08	55.21	0.52	45.9
	Interaction	NS	*	NS	*	*

a: Relative growth rates calculated on the basis of shot dry weight until anthesis; b: Leaf area, leaf water potential (LWP) and root dry weight were recorded at maize anthesis; * Significant at P=0.05; NS: Non-significant

Most smallholders in developing nations grow maize for seed, and hence the benefits of management strategies need to be reflected in seed yields. The improvements gained both in germination and vegetative growth due to improved fallows resulted in enhanced yield components and yields (Table 5). The impact of the improved fallows on kernel rows per cob was similar in both seasons (10–11%) compared with natural fallow. In contrast, seed weights were increased by 16% due to improved fallows in the minor season and by 9% in the major season, compared to natural fallow. This again highlighted the importance of the organic matter added through improved fallows at the beginning of the minor season, when crop growth is affected adversely by the dry conditions.

The most significant impact of the improved fallows was observed in the grain yield (Table 5). The yield of maize in the major season was significantly greater, and the impact of the improved fallows was hence less evident. Improved fallows with *Crotalaria* and *Tithonia* increased seed yields by 20% and 23%, respectively, compared with natural fallow in the major season, resulting in significantly enhanced harvest indices, illustrating the greater partitioning of dry matter into the grains, the economic product. In the minor season, when the overall yields were lower, *Tithonia* and *Crotalaria* fallows increased maize seed yields over that in the natural fallow plots by 42% and 34%, respectively, again increasing the harvest indices. This also suggests that the impact of *Tithonia* was greater in terms of increasing the yield of maize in the minor season. However, the impact of the improved fallows on the harvest indices was greater in the major season, suggesting that although yields were increased to a greater extent in the minor season, the partitioning of photosynthates to maize seeds in the improved fallow plots was more efficient in the major season. The results also clearly showed that the impact of *Tithonia* as a natural fallow was greater on the seed yield of maize in both seasons compared to the benefits accrued by the incorporation of *Crotalaria* as a green manure, another phenomenon that needs elucidation under field conditions in tropical Asia, where legumes are considered the normal green manures.

Table 5

Yield components and yields of maize as affected by fallows in major and minor seasons

Season	Fallow	Kernel rows cob^{-1}	1000-seed wt (g)	Yield (kg ha^{-1})	Harvest index
Major	*Crotalaria*	15.2	245	4382	0.45
(Dry fallow)	*Tithonia*	15.9	239	4491	0.43
	Natural	13.8	224	3645	0.39
Minor	*Crotalaria*	13.8	185	2545	0.36
(Wet fallow)	*Tithonia*	13.5	182	2709	0.37
	Natural	12.4	159	1899	0.32
LSD(P=0.05)	Fallow	2.95	10.8	213.5	
	Season	1.51	30.4	399.8	
	Interaction	*	NS	*	

* Significant at P=0.05; NS: Non-significant

Conclusions

The study on the impact of improved fallows in the major and minor seasons on the productivity of maize under field conditions indicated that in the major season, when rainfed agriculture is possible, growing an improved fallow such as *Crotalaria* or *Tithonia* instead of an arable crop has a significant impact on the succeeding maize crop in the minor season. Although farmers do not generally adopt this system, fields left fallow in the minor season under normal conditions could easily be used for growing a green manure, especially *Tithonia*, which would have a beneficial impact on the maize crop of the major season, and possibly help develop a sustainable system through the addition of quality organic matter to the soil, rather than incorporating the aggressive weeds that grow in this season, which could be a problem in the succeeding major season. This also shows that the results obtained in African smallholder farming systems on the use of fallows for increasing maize yields (e.g. Whitbread et al., 2004) can be applied for smallholder systems in tropical Asia, where rainfall is greater.

Another possible option available to farmers who cultivate crops in the major season is to use the period of approximately three months (late January – late April) in between the two seasons, which is an inter-monsoonal period, to grow a green manure crop using residual moisture in the soil, rather than leaving fields under normal fallow conditions. This would ensure the addition of a significant quantum of better quality green manure to the fields at the onset of the minor season, and thus have a beneficial impact on maize yields. This, as suggested by Van Noordwijk (1999), would also become a mosaic within a sequential system of spatially interfacing and interacting crops and fallow periods to optimize resources, especially soil moisture and nutrients, which are the limiting factors of crop productivity in the small upland farms of Asia. Research is in progress to compare the benefits of these inter-seasonal fallows on the productivity of a succeeding maize crop in major and minor seasons.

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TEMPERATURE DEPENDENCE OF WHEAT DEVELOPMENT

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Among the abiotic stress factors influencing the growth and productivity of wheat varieties, extremely high temperatures have the most limiting effect. In an experiment set up in the gradient chamber of the Martonvásár phytotron to test the effect of various temperatures on four winter wheat varieties and one variety of spelt, substantial differences were observed in the heat stress tolerance of the varieties. There was a considerable reduction in the number of shoots and spikes as the result of heat stress, leading to a drastic loss of grain yield. It was clear from changes in the biomass and in the grain:straw ratio that extremely high temperatures led to a substantial reduction in the ratio of grain to straw in the varieties tested. In response to high temperature the wheat plants turned yellow earlier due to the rapid decomposition of the chlorophyll content. This resulted in a considerable shortening of the vegetation period and early ripening. Reductions in the parameters tested were observed at different temperature levels for each variety, indicating considerable differences in the ability of the varieties to adapt to abiotic stress factors.

Key words: heat stress, yield, grain:straw ratio, chlorophyll content

Introduction

Temperature, which has a considerable influence on plant development, is one of the most variable environmental components. As the result of climate change, research on the damaging effect of extremely high temperatures on field crops has become increasingly topical. Wheat plants develop most favourably at a temperature of 10–24°C. Each 1°C increase in temperature above the optimum between the end of tillering and grain filling causes an average 4% loss of yield (Rascio et al., 1988). Higher mean temperatures speed up plant development, resulting in a shorter vegetation period for field crops. Aging processes accelerate in very hot conditions and the grain-filling period of cereals becomes shorter, leading to considerable yield losses. Hot weather during grain filling leads to a substantial reduction in grain number and grain size, again causing yield losses (Blumenthal et al., 1995; Wheeler et al., 1996). Due to the physiological changes induced by heat and drought stress there is a rapid decline in the relative leaf water content and in the leaf chlorophyll content, causing the plants to turn yellow far earlier than the control plants, while the grains reach harvest ripeness more rapidly (Jiang and Huang, 2001; Bencze et al., 2005). Under the climatic conditions of Hungary, Varga (2007) found that thermal factors had a greater influence than hygric factors. On average a 1–1.5°C increase in temperature leads to a 1–2 week shortening of the vegetation period. Higher temperatures also result in a reduction in the spike number (Oh-e et al., 2004).

It must not be forgotten, however, that changes in the meteorological system always have a complex effect and that other changes, such as water deficiency, may aggravate the unfavourable effect of heat stress. Phenological and yield data indicate that plants are most sensitive to drought from flowering to milky ripeness, but are also very sensitive between booting and heading and moderately sensitive from shooting to booting. Drought stress during these sensitive stages has been found to result in yield losses equivalent to those caused by constant water deficiency (Németh et al., 2005).

It is not sufficient to simply determine the damaging effect of high temperature in general. It is important to measure stress effects at various temperatures in order to determine the borderline temperature below which the plants are able to adapt sufficiently to survive the damage and produce an acceptable yield and above which they respond with significant losses of yield, biomass and harvest index. The aim of the experiments was to study the adaptability of various wheat varieties at different temperatures and to interpret the responses induced by different genetic backgrounds.

Materials and methods

The gradient chamber constructed in the Martonvásár phytotron allows plants to be tested over a range of temperatures simultaneously and the complex interactions existing between temperature and wheat development to be investigated (Tischner and Veisz, 1996; Kőszegi and Kovács, 2003). The plants were arranged on the growth bench with twelve varieties in the columns and twelve temperatures in the rows. The results achieved for five of the varieties will be discussed in detail, as these represented the most distinct reaction types. These varieties were Plainsman V. (USA), Mv Mambó, Mv Mariska and Öthalom from Hungary and the German spelt variety Frankenkorn. The plants were transferred to the gradient chamber from a homogeneous (PGV) growth chamber when they reached the booting stage. This stage was reached on different dates for the individual varieties (Table 1). The plants were then kept at various levels of temperature stress until harvest. The temperatures in the rows ranged at 1°C intervals from 20–30°C (night) and from 25–35°C (day), with a 16-hour daylength (Tischner et al., 1997). The illumination was placed at an average 40 cm from the plant tips at a constant angle. The plants were given different amounts of water to achieve optimum moisture content under the differing water consumption and transpiration conditions created at the various temperatures.

During the heat stress treatment, measurements were made on the chlorophyll content, and after maturity a record was made of the shoot number, spike number, plant height, grain yield and biomass per plant and of the harvest index, grain number and thousand-kernel weight. The results were evaluated in comparison with the variety mean.

Table 1
Varieties tested in the temperature gradient chamber and the number of days from sowing to the start of the treatment

Varieties tested	No. of days from sowing to the start of the treatment
Plainsman V. (USA)	61
Mv Mambó (H)	64
Mv Mariska (H)	61
GK Öthalom (H)	65
Frankenkorn (D)	79

Results

The changes recorded in morphological traits exhibited a wide range. At the temperature levels tested, the experimental parameters exhibited polynomial changes as a function of the environmental factors. In some temperature ranges significant differences were detected. Parallel with the rise in temperature there was a substantial decrease in the plant height, shoot number and spike number. The shoot number was influenced to the greatest extent in Plainsman V. and Frankenkorn (Fig. 1), where the number declined steeply. The varieties GK Öthalom and Mv Mambó formed a separate group, where the responses to environmental factors were less intensive. The spike number exhibited the most drastic reduction in Mv Mariska and Frankenkorn (Fig. 2), while the least change was observed for Mv Mambó, as indicated by the mild slope of the polynomial. The gradual increase in temperature also had a substantial influence on the plant height (Fig. 3), with a clearly perceptible reduction in the height of all the varieties as the heat stress increased.

As the temperature rose there was a gradual decline in the chlorophyll content of the plants, the only exception being the spelt variety Frankenkorn, which exhibited the best tolerance of high temperature throughout the experiment. For this variety, the almost horizontal slope is indicative of the lack of any correlation between the two factors, i.e. temperature did not play a decisive role in the level of the chlorophyll content in Frankenkorn, in contrast with the other varieties tested (Fig. 4).

In response to the increasing temperature gradient, the genotypes exhibited various extents of biomass reduction (Fig. 5). At lower temperatures the greatest biomass was recorded for Mv Mariska and Frankenkorn. GK Öthalom also had relatively high biomass, and this variety continued to produce a stable high level of biomass even at higher temperatures, where the other varieties responded with a drop in biomass.

Depending on their heat tolerance, the wheat varieties exhibited the greatest sensitivity in varying temperature ranges. This was manifested primarily as a loss of yield (Fig. 6). Some varieties responded with lower grain yield at a temperature of 29–30°C (Frankenkorn, Plainsman V.), while GK Öthalom only exhibited yield loss at 32°C. For Mv Mambó and Mv Mariska significant yield reductions were recorded at 31°C.

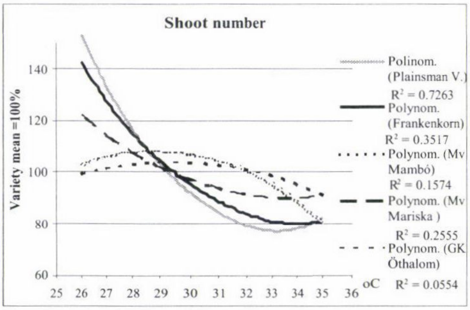


Fig. 1. Changes in shoot number at different temperature levels as a percentage of the variety mean

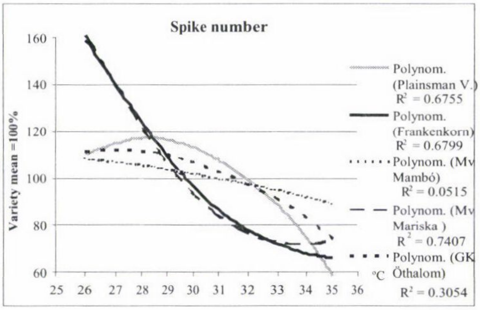


Fig. 2. Changes in spike number at different temperature levels as a percentage of the variety mean

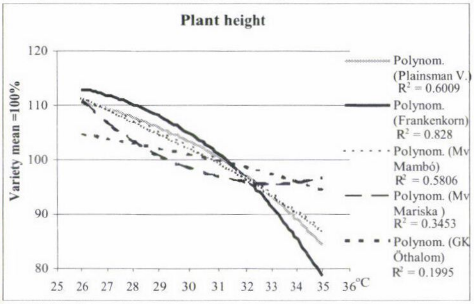


Fig. 3. Changes in plant height at different temperature levels as a percentage of the variety mean

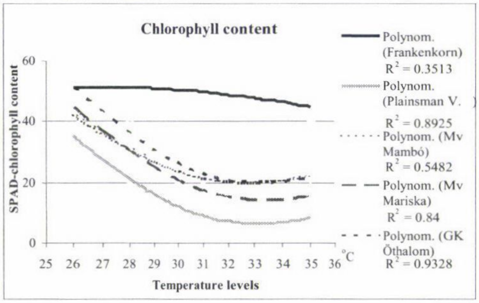


Fig. 4. Changes in the chlorophyll content, averaged over the measuring dates

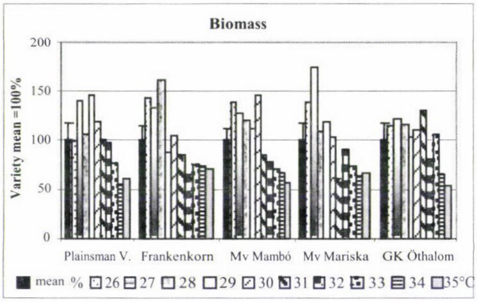


Fig. 5. Changes in biomass at various temperatures, as a percentage of the variety mean

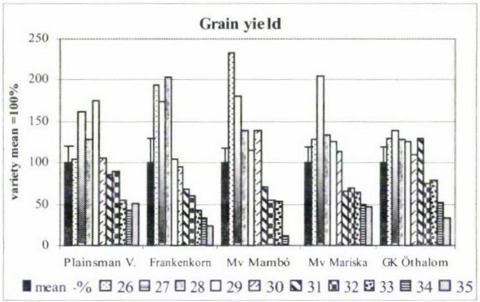


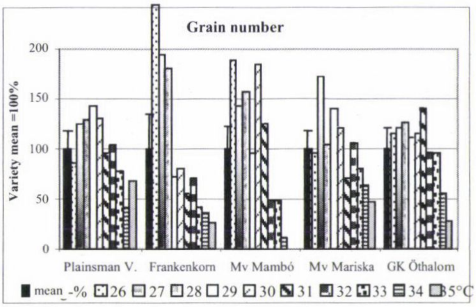
Fig. 6. Changes in grain yield at various temperatures, as a percentage of the variety mean

Similar reductions were observed in the grain number as a result of increasing temperature. Under normal conditions the highest grain numbers were recorded for Frankenkorn and Mv Mambó, and the latter was capable of producing a high grain number even at extremely high temperatures, but in the case of Frankenkorn a drastic reduction was observed even at 29°C. GK Öthalom and Plainsman V. proved the most tolerant of temperature change in this respect, while Mv Mariska responded with a decline in the grain number from 31°C and Mv Mambó from 32°C (Fig. 7).

Significant changes in the thousand-kernel weight (TKW) were observed primarily at temperatures of 31–32°C. At other temperatures, both increases and decreases in TKW were observed. In the case of Frankenkorn and Plainsman V. there was a significant reduction in TKW from 26–35°C, while two varieties, GK Öthalom and Mv Mambó, exhibited a slight increase in TKW between 33 and 35°C, probably in response to extremely high temperature stress from a very early stage of development (from booting to harvest) (Fig. 8).

Reductions of varying extent were observed in the harvest index (Fig. 9). Some varieties exhibited less sensitivity to increasing temperature for this parameter (Plainsman V., Mv Mariska and GK Öthalom), but Mv Mambó responded with a considerable decline in the harvest index.

The total aboveground biomass was analysed in terms of two parameters, the total grain mass and the total straw mass. For all five varieties it was observed that high temperature caused a reduction not only in the total biomass, but also in the grain:straw ratio (Fig. 10).



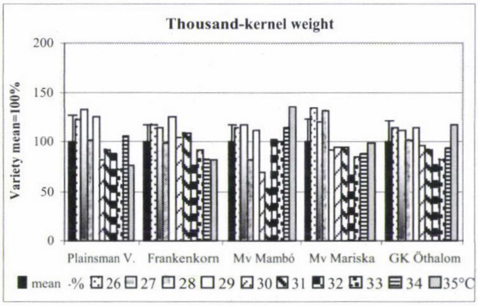


Fig. 7. Changes in grain number at various temperatures, as a percentage of the variety mean

Fig. 8. Changes in thousand-kernel weight at various temperatures, as a percentage of the variety mean

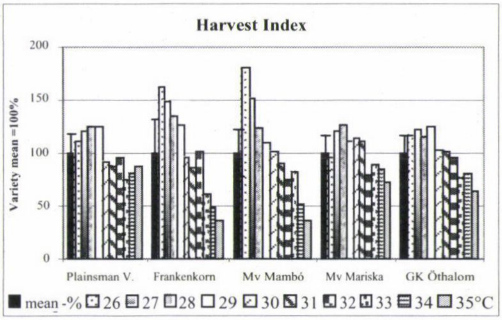


Fig. 9. Changes in harvest index at various temperatures, as a percentage of the variety mean

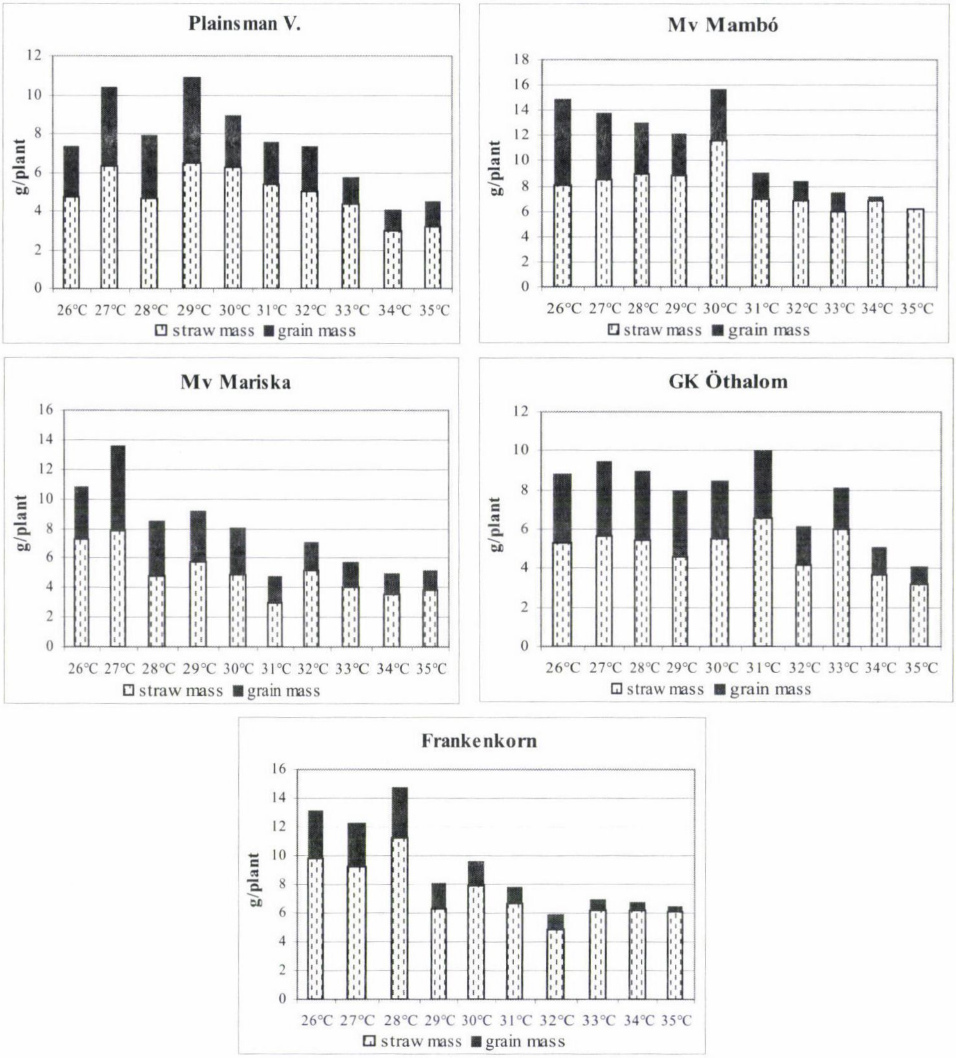


Fig. 10. Changes in the grain:straw ratio in the aboveground biomass of five varieties in response to an increasing temperature gradient

Discussion

Experiments carried out in a gradient chamber proved that temperature plays a decisive role in the development and productivity of plants. Wheat plants grown at 28°C from booting to full maturity produced substantially better yields than those exposed to a temperature of 35°C. The present results confirm the findings of Jiang and Huang (2001) and Bencze et al. (2005), who observed that plants grown at high temperature started to turn yellow more rapidly due to a gradual decline in the chlorophyll content, suggesting a drastic reduction in the photosynthetic activity. The stress applied in the experiment resulted in accelerated ripening. There was also a reduction in the spike number in response to high temperature, as also reported by Oh-e et al. (2004). With the exception of a few varieties that were more tolerant of heat stress, the majority of the wheat genotypes exhibited a drastic reduction in yield parameters as the result of long-term stress. The most sensitive varieties had yield levels of 12–24% when grown at 35°C, while the most resistant were able to produce yields of around 50% the normal level. An analysis of total biomass and the grain:straw ratio showed that extremely high temperatures caused a substantial reduction in grain mass compared to the straw mass in the varieties tested. A comparison of all the agronomic parameters examined revealed that TKW was the least affected by high temperature. The grain shrinkage associated with drought stress was not observed, or only to a lesser extent, as a result of heat stress. Reductions in the experimental parameters could be observed from various temperature levels for the individual varieties. Yield losses were recorded by a temperature of 29°C for some varieties, while for others drastic yield reductions were not observed until the temperature reached 32°C. In general, varieties suited to a cooler climate, such as Frankenkorn, were only able to achieve their high yield potential at lower temperatures. GK Öthalom, on the other hand, which was unable to produce a yield much greater than that of Frankenkorn at lower temperatures, exhibited a much less severe reduction in yield at high temperature than the other varieties. It could be seen from the results that the varieties exhibited considerable differences in heat stress tolerance when grown under identical conditions, indicating that a high level of genetic variability exists in terms of tolerance to abiotic stress factors.

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STUDIES ON THE DROUGHT AND HEAT STRESS RESPONSE OF GREEN BEAN (*Phaseolus vulgaris* L.) VARIETIES UNDER PHYTOTRONIC CONDITIONS

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The drought tolerance of six green- and yellow-podded varieties of green beans with different genetic backgrounds was tested in the phytotron. During the week prior to flowering the plants were kept either at 25/15°C (day/night) or at high temperature (30/15°C), with RH 75% and optimum water supplies. The heat-stressed plants were then divided into three groups; the first was returned to the control (25/15°C) chamber (RH 75%, optimum water supplies), while the second and third were exposed to mild drought stress (RH 60%, 50% water) at temperatures of 30/15°C and 35/25°C, respectively, throughout the flowering period.

The varieties survived the short period of heat stress (30/15°C) prior to flowering without damage provided the temperature during flowering was reduced to 25/15°C and the water supplies were optimum. There was a sharp increase in the carotene level in the leaves of drought-stressed plants when the temperature during flowering was 30/15°C, but in plants exposed to 35/25°C during flowering the level dropped to near the control level. The latter group exhibited considerable damage, with a reduction in the water-soluble antioxidant content (ACW: antioxidant capacity of water-soluble substances) and the chlorophyll *b* content compared with the control.

The antioxidant content (ACW) in the dark green leaves of green-podded varieties was lower than in the yellow-podded varieties and did not change as the result of drought and heat stress. In yellow-podded varieties, however, there was a significant decline in ACW in response to stress. Differences between the varieties in their adaptability to drought and heat could be detected as changes in the chlorophyll and carotene contents of the leaves even at 30/15°C.

Key words: green beans, drought stress, antioxidants, ACW, phytotron

Introduction

One of the most important environmental factors influencing plant productivity at present is global climate change, involving the increased frequency of extreme weather conditions, including periods of high temperature and drought. In the course of their development bean plants are able to tolerate high temperature, but water is the major limiting factor for flower and seed formation. During long periods of dry weather, the lack of soil moisture leads to a reduction in photosynthesis and an increase in transpiration. Plant water deficiency is indicated when the temperature of the foliage is higher than that of the air. Lower soil moisture and higher radiation levels result in greater positive temperature differences (Helyes and Varga, 1990; Helyes et al., 2006). The drop in the relative water content of bean leaves is greatest at noon, and the magnitude of the decrease is correlated with the pod number, seed size and seed yield, as reported by Omae et al. (2005). The great genetic variation observed in the magnitude of this decrease allows selection to be made between the varieties for drought tolerance.

Many authors treat water deficiency stress as a form of oxidative stress (Dhindsa and Matowe, 1981; Burke et al., 1985), as the changes caused in the cell membranes as the result of lipid peroxidation are similar to those occurring during dehydration. Xing et al. (2001) induced water deficiency stress in pea seedlings using PEG solution, and demonstrated that the level of lipid peroxidation gradually increased, leading to the formation of free radicals that damaged the plasma membrane. The young leaves of pea were more resistant to oxidative stress than older leaves, due to their greater superoxide dismutase (SOD) enzyme activity (Donahue et al., 1997). The differences in water deficiency tolerance observed between bean varieties could be attributed to differences in the activity of the SOD and catalase enzymes (Türkan, 2005).

Plants contain antioxidant enzymes capable of converting light energy into chemical energy. Catalase and superoxide dismutase (SOD) are considered to be the most active in this process, being able to convert superoxide radicals and hydrogen peroxide (H_2O_2) into water and molecular oxygen (Scandalios, 1993). In addition, numerous, as yet unidentified water-soluble compounds with an antioxidant effect also play a role in plant responses aimed at overcoming environmental stress factors. It is possible that differences in the total quantity of antioxidant compounds give a better indication of the level of plant adaptability or stress tolerance than the presence of a single compound or enzyme and may provide a better basis for the testing of basic breeding materials. Few studies have been made on the drought stress response of bean plants, especially with respect to antioxidant compounds.

In the present work the effect of drought and heat stress during the flowering of green beans was investigated in order to detect differences in the adaptability of the varieties.

Materials and methods

The drought and heat stress responses of six green bean varieties of different genetic origin and pod type (yellow-podded: Hungold, Maxidor and Debreceni Sárga; green-podded: Buvet, Zsófi and Masai) were investigated under controlled conditions. The varieties were sown in pots in the greenhouse. Each treatment consisted of six pots, each containing two plants. The plants were given satisfactory water supplies until the appearance of the green buds. At the age of 29 days the plants were transferred to Conviron PGR-15 climatic chambers in the phytotron of the Agricultural

Research Institute of the Hungarian Academy of Sciences in Martonvásár. Control plants (code: Cont.) were maintained at day/night temperatures of 25/15°C with high humidity (RH 75%) and optimum water supplies both before and during flowering. Treated plants were kept at high temperature (30/15°C) with optimum water supplies for a week before flowering, after which some were returned to the control (25/15°C) chamber with optimum water supplies (code: 25/15), while the remainder were exposed to mild drought stress (50% water, RH 60%) combined with mild (30/15°C) or severe (35/25°C) heat stress throughout the flowering period (codes: 30/15 and 35/25, respectively).

For leaf analysis, the middle leaf at the third node was removed from both plants in each plot (representing two replications). The leaf samples were weighed and stored in liquid nitrogen prior to chemical analysis. Samples were taken in a similar manner at flowering, except that all three leaves were removed from the fourth node, due to the smaller leaf size. An analysis was made of the chlorophyll and carotene contents of the leaves in mmol/g (Hendry and Price, 1993). The photochemiluminescence method (Popov and Lewin, 1999) was used to determine the quantity of water-soluble antioxidants (given as μg/mg ACW, antioxidant capacity of water-soluble substances). The measurements were carried out on lyophilised samples using a PHOTOCHEM (Analytik Jena Ag, Germany) chemiluminometer in the laboratory of the Horticultural and Plant Biotechnological Department, Centre for Agricultural and Engineering Sciences, University of Debrecen.

The data were evaluated by analysis of variance using the SPSS program.

Results

Even with optimum water supplies, a 5°C rise in temperature (30/15°C) during the period prior to flowering resulted in a substantial (26.3%) reduction in the mean leaf mass, while the chlorophyll and carotene contents rose significantly. There was no change in the quantity of water-soluble antioxidants, however, compared to the control (25/15°C) (Table 1). During flowering, when this 5°C rise in temperature was associated with water deficiency there was a further decline in the mean leaf mass, while the carotene content continued to be high compared to that of the control plants. The data indicated that a short period of mild drought and heat stress during the flowering period of bean did not influence either the chlorophyll content or the antioxidant content of the leaves (Table 1).

Table 1

Effect of temperature and water deficit on the leaves of French bean

Period	Temperature °C	Average leaf weight (g)	Chlorophyll *a* mmol/g	Chlorophyll *b* mmol/g	Total chlorophyll mmol/g	Carotene mmol/g	ACW μ/mg
Before flowering	25/15^1	0.43 a	26.34 b	3.93 b	30.27 b	26.42 b	14.93 a
	30/15^1	0.34 b	37.34 a	5.69 a	43.02 a	36.61 a	14.18 a
	Difference %	–26.30	41.70	44.60	42.10	38.50	–5.1
During flowering	25/15^1	0.37 b	34.13 a	5.19 a	39.31 a	28.23 b	10.29 b
	30/15^2	0.15 c	35.20 a	5.89 a	41.09 a	38.70 a	7.26 b
	Difference %	–61.00	3.10	4.50	4.50	37.10	–30.00

^{1} = optimum water supplies; ^{2} = water deficit; Values in each column having different letters are significantly different at the P<0.05 level using Duncan's multiple range test

All the varieties adapted well, with no visible leaf damage, to a short period of higher temperature (30/15°C) prior to flowering, providing the temperature dropped to 25/15°C with optimum water supplies during flowering itself. The ACW content in the leaves of these plants (code: 25/15) was only slightly lower than that of the control plants, while the leaf mass was significantly reduced and no change was observed in the chlorophyll and carotene contents (Table 2).

In plants which remained at 30/15°C and were exposed to water stress during flowering (code: 30/15) only the carotene level in the leaves rose substantially compared to the control, indicating that the plants endeavoured to overcome drought and heat stress through morphological changes, such as a reduction in the leaf mass, and an increase in the carotene content. The quantity of water-soluble antioxidants (ACW) in the leaves was slightly lower in these plants compared with the control plants grown at 25/15°C.

When the temperature was further increased to 35/25°C plant growth ceased and the leaves started to turn yellow, after which the plants dropped a large proportion of their leaves and flowers. A combination of high temperature (35/25°C) and water deficiency during flowering led to a sharp decline in the chlorophyll *b* and ACW contents compared with the control (Table 2). The plants responded to water deficiency and high temperature during flowering with a reduction in leaf size, while the large-leaved varieties lost most of their leaves and flowers.

The yellow-podded variety Debreceni Sárga has large, yellowish green leaves, while Hungold and Maxidor have mid-green leaves of medium size. All the green-podded varieties (Buvet, Zsófi, Masai) have dark green leaves. The lowest contents of chlorophyll *a* (22.33 mmol/g), chlorophyll *b* (3.2 mmol/g) and carotene (19.7 mmol/g) were recorded for the Debreceni Sárga variety, which had the most sensitive response to a rise in temperature; at 30/15°C there was a considerable reduction in the chlorophyll *a* component compared to the control (Table 3). At higher temperature (35/25°C) combined with water deficiency there was no great change in the chlorophyll *a* quantity compared with the control and no difference was detected between the varieties. At this high temperature the greatest chlorophyll *b* content was detected in the leaves of Masai (6.22 mmol/g), and a substantial decrease compared to the control was only recorded for Maxidor (Table 3).

Table 2

Effect of drought stress on the leaves of French beans during flowering

Treatment code	Average leaf weight (g)	Chlorophyll *a* mmol/g	Chlorophyll *b* mmol/g	Total chlorophyll mmol/g	Carotene mmol/g	ACW μg/mg
Cont.	0.37a	34.13a	5.19a	39.31a	28.23b	10.29a
25/15	0.30b	35.39a	5.57a	40.96a	29.48b	6.95ab
30/15	0.19cd	35.20a	5.89a	41.09a	38.7a	7.26ab
35/25	0.15d	31.60ab	4.57b	36.18ab	25.80b	5.37b

Values in each column having different letters are significantly different at the $P<0.05$ level using Duncan's multiple range test; For treatments, see Materials and Methods

Table 3

Reactions of French bean cultivars to drought stress during flowering

Compound	Treatment code	Yellow-podded cultivars			Green-podded cultivars		
		Hungold	Maxidor	Debr. Sárga	Buvet	Zsófi	Masai
Chlorophyll *a* mmol/g	Cont.	29.22 ab	39.47 a	22.33 b	34.40 ab	33.83 ab	45.54 a
	25/15	31.85 b	23.20 b	30.00 b	50.65 a	38.70 ab	37.95 ab
	30/15	41.22 ab	35.69 ab	16.66 c**	40.47 a	35.84 ab	41.33 a
	35/25	27.50 ab	24.85 ab	25.55 bc	37.05 ab	33.90 ab	40.77 ab
Chlorophyll *b* mmol/g	Cont.	4.26 c	5.70 ab	3.20 c	5.59 ab	5.13 bc	7.24 ab
	25/15	4.79 bc	4.90 bc	4.41 cb	7.07 a	6.27 ab	5.99 b
	30/15	6.40 b **	5.62 ab	2.44 c	6.47 ab	5.79 bc	8.64 a
	35/25	4.15 c	3.59 c **	3.81 c	5.54 bc	4.14 c	6.22 b
Carotene mmol/g	Cont.	23.85 cb	31.90 abc	19.70 c	29.15 bc	27.75 bc	37.00 b
	25/15	27.65 b	20.30 c	29.40 bc	38.10 ab	32.65 bc	28.80 b
	30/15	42.05 a **	42.55 a	22.30 c	41.68 ab	31.58 bc	52.06 a**
	35/25	22.50 cb	21.80 cb	22.70 cb	31.30 b	24.30 bc	32.20 b
ACW μg/mg	Cont.	20.65 a	11.95 b	18.60 a	1.61 cd	3.01 c	5.91 c
	25/15	8.75 bc**	6.30 c**	10.44 b**	4.72 cd	4.80 cd	6.69 bcd
	30/15	10.55 b**	11.06 b	7.81 bc**	3.86 cd	4.03 cd	6.21 c
	35/25	9.90 b**	7.67 b	6.76 b**	1.91 cd	3.12 cd	2.89 cd

Debr. Sárga: Debreceni Sárga; ACW: water-soluble antioxidants; Values in each row and column having different letters are significantly different at the $P<0.05$ level using Duncan's multiple range test; **: Significantly different from the control at the $P<0.05$ level; For treatments, see Materials and Methods

Although there was a rise in the carotene level in the leaves of all the varieties at 30/15°C compared to the control, the difference was only significant for the yellow-podded variety Hungold and the green-podded variety Masai. In response to a further rise in temperature the carotene level in the leaves declined to approximately the control value.

In the dark green leaves of the green-podded varieties the antioxidant (ACW) content was lower than in the yellow-podded varieties and did not change as a result of drought and heat stress (Table 3). Yellow-podded varieties exposed to a temperature of 30/15°C prior to flowering exhibited a substantial decrease in the ACW content when they were returned to optimum conditions (25/15°C) in the control chamber during flowering (code: 25/15), suggesting that even a slight change in the temperature caused a more sensitive response in these varieties than in the green-podded variants. When the temperature was increased further, the ACW level in the leaves only remained significantly lower than the control level in the varieties Hungold and Debreceni Sárga, while no change was observed for the green-podded varieties.

Discussion

Beans, particularly green beans, cannot be classified as drought-tolerant plants. The growth of plants exposed to water deficiency is inhibited, resulting in a reduction in the leaf surface and pod mass (Nemeskéri, 1990). A complex regulatory mechanism involving enzymes, metabolites and special proteins, is known to exist in plants as a defence against drought stress (Györgyey, 1999; Páldi et al., 1998). This is confirmed by the decreased activity of the proline oxidase enzyme in common bean plants exposed to drought stress compared to irrigated plants (Barron and De-Mejia, 1998). The difference observed in the tolerance of water deficiency in the species *Phaseolus vulgaris* and *P. acutifolius* can be attributed to the activity of enzymes with an antioxidant effect (SOD, catalase). In *P. vulgaris* these enzymes have low activity, making the plants extremely sensitive to water deficiency (Türkan, 2005).

In order to test whether there were differences in drought and heat tolerance between varieties within the *P. vulgaris* species, various groups of compounds responsible for adaptability were extracted from the green leaves and analysed. The aim of the studies was not to detect the individual enzymes or other compounds reported in the literature to be active in stress responses. It is thought that, in addition to the antioxidant enzymes, the joint effect of numerous other, as yet undetected water-soluble antioxidant compounds may be responsible for the adaptation of plants to environmental stress factors. These compounds react with free radicals and neutralise them, thus overcoming the damaging effects of stress.

Changes in the total quantity of water-soluble antioxidants (ACW) and leaf pigments were monitored in bean plants grown in the phytotron with various levels of drought and heat stress. A 5°C rise in the daytime temperature prior to flowering, in the green bud stage, did not cause any substantial change in the pigment quantity in the leaves, provided water supplies were satisfactory. When increased temperature during flowering was combined with water deficiency, however, there was a drop in the leaf mass. Similar results were obtained in previous studies (Nemeskéri, 1990; 2001).

The bean yield was influenced primarily by the climatic conditions during flowering, which is the most sensitive phase of plant development. A short period of mild drought and heat stress during flowering had no significant effect on the chlorophyll and antioxidant quantities in the leaves, and thus on plant development (Table 1).

Plants grown for a lengthy period at a temperature of 30/15°C before and during flowering endeavoured to overcome the effects of drought and heat stress by means of morphological changes, such as a reduction in leaf size and mass, and by an increase in carotene synthesis (Table 2). Under these conditions there was a slight reduction in the antioxidant (ACW) content of the leaves compared with control plants grown at 25/15°C with optimum water supplies. It thus appears that the water-soluble antioxidants do not play a major role in the adaptability of bean plants to drought and heat stress.

According to Iturbe-Ormaetxe et al. (1998) a severe water deficit resulted in the almost complete inhibition of photosynthesis and reduced the quantities of chlorophyll, beta carotene, neoxanthine and lutein in the leaves. Further damage was caused by the conversion of violaxanthine to zeaxanthine. In the present experiment the combination of water deficiency with increased temperature had a similar effect. Drought-stressed plants grown at 35/25°C exhibited severe damage, with a cessation of growth and the yellowing of the leaves due to the decomposition of the chlorophyll *b* content, followed by an intensive loss of leaves and flowers.

If the plants are exposed to drought and heat stress during flowering, the biochemical defence mechanisms (e.g. ACW, carotene formation) are soon exhausted. Contrary to expectations, the ACW level in green bean leaves declined in response to drought and heat stress. This reduction was particularly pronounced in yellow-podded varieties compared with the control, indicating that these varieties respond sensitively to high temperature and water stress during flowering. By contrast, in the leaves of green-podded varieties, with the exception of Buvet, there was no change in the low ACW level at a temperature of 30/15°C. The results suggest that varieties where the chlorophyll *a* content is dominant over chlorophyll *b* have better tolerance of temporary drought and heat stress.

Conclusions

A short period of higher temperature (30/15°C day/night) before flowering, combined with mild drought stress during flowering, induced a substantial increase in the carotene content in bean leaves, but there was no great change in the chlorophyll or antioxidant contents compared with the optimum temperature of 25/15°C. The results showed that chlorophyll *b* was more sensitive to drought and heat stress during flowering (35/25°C) than chlorophyll *a*. At 35/25°C a considerable reduction in the quantity of water-soluble antioxidants (ACW) could be observed in the leaves compared with the control plants. At a temperature of 30/15°C, which often occurs under field conditions, differences in the adaptability of the varieties to drought and heat stress could be detected as changes in the chlorophyll and carotene contents of the leaves. Further studies will be required to determine the role of antioxidants in the drought stress response of bean varieties.

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EFFECT OF DROUGHT DAMAGE IN VINE VARIETIES

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Drought stress caused substantial damage to the certified vineyard examined in Szomód, Hungary, in 2004. The varieties included in the experiment were Chardonnay, Sauvignon Blanc, Királyleányka, Pinot Noir and Kékfrankos. Drought caused the greatest deterioration in yield quantity and quality in the varieties Kékfrankos and Királyleányka. The yield was 66% lower and the quality 3 MM° (Hungarian must degrees) lower than that recorded on the control area, which was less severely affected by the drought. In the case of Pinot Noir the soil water-holding capacity (WHC%) was below the critical 15% value, again leading to a decline in yield quantity and sugar concentration, though the difference compared with the control was not as great as for the two former varieties. Although the yield of Chardonnay decreased slightly, the quality improved. This could probably be attributed to the similarly low soil WHC% in the control and drought-struck areas.

All in all it could be concluded that all the vine varieties exhibited stress symptoms at a soil WHC of below 30%. The varieties could be divided into three groups on the basis of stress sensitivity, with Királyleányka and Kékfrankos in the most susceptible group, Chardonnay in the moderately susceptible group, and Pinot Noir and Sauvignon Blanc intermediate between the two.

Key words: drought, soil water-holding capacity, moderate and extreme water deficiency, Hungarian must degrees, vine (*Vitis vinifera* L.)

Introduction

Many authors have provided definitions of drought. The simplest definition is that given by the World Meteorological Organisation (WMO, 1986): drought is an above-average water deficiency. Petrasovits (1989) described agricultural drought as a long-term water deficiency on crop production areas, making water the most limiting of all the environmental factors.

Water is one of the most important components of plant organisms, without which there is no protoplasm and no metabolism. The coordination of the functions of the roots, stem and leaves ensures that the water balance in the plant remains more or less in equilibrium throughout the life of the plant. Life processes only take place normally in plant cells containing a sufficient quantity of water. Water also transports nutrients and the assimilates formed in the course of the metabolism. Water is required for osmosis and for the development of turgor pressure. The great evaporation heat of water is an important factor in temperature regulation. As water is constantly transpired, the plant needs to replace it from the soil, which means that in optimum cases plant water uptake is constant and continuous (Szalai, 1994).

When the rainfall distribution is unfavourable, the quantity of water essential for these processes may not be available, but plants are able to survive a 10–20% water deficit without any substantial damage. If, however, the relative humidity of the air is low and the soil water reserves are exhausted, the plant will wilt and the leaves will wither and drop. In wilting plants, disturbances arise in the metabolism, leading to decomposition rather than synthesis. When the transpiration from young leaves is excessive, the young roots are deprived of water, causing them to lose contact with the soil and die. When the water supply is restored, regeneration may take place, but this takes a considerable time, and may result in loss of all or part of the yield. As the water content declines there is generally a retardation, or complete cessation, of synthetic processes, and in extreme cases the cells may die.

Plants have developed a number of mechanisms that allow them to adapt to unfavourable environmental factors, either phylogenetically or in the course of ontogenesis (Pethő, 1998).

Due to their origin, vines have a relatively good tolerance of drought. This can be attributed to the thick leaf cuticle and rapid stomatal closure, activated by abscisic acid. Vines have a water requirement of approx. 400–500 L/m^2 during the growing season. As drought becomes more severe, the vines use water more economically, whereas in the case of excessive supplies they produce relatively less dry matter (luxury water utilisation). In general the water utilisation of vines is adjusted to the amount available, but long-term water deficiency should be avoided (Bauer, 2004).

The question of what level of drought vines are capable of surviving without permanent damage, and when the process becomes irreversible, is of more concern today than it has ever been. It is influenced by numerous factors, including the soil structure, the local meteorological conditions, the vine variety, the rootstock, the age and spacing of the vineyard, phytotechnical operations, the crop load, etc. In the droughty year of 2004 examinations were made on the responses of various vine varieties to water deficiency when grown under the same environmental and technological conditions but with heterogeneous soil conditions.

Materials and methods

The experiments were carried out in summer 2004 on a 20.5-hectare certified vineyard in Szomód, Hungary. The varieties and clones included in the experiments were as follows: Chardonnay Bb.75/1, Sauvignon Blanc Bb.297/1, Királyleányka 21, Pinot Noir M2 and Kékfrankos Kt. 1; rootstock: B × RT 5 BB. The following descriptions of the production value of the varieties/clones are based on Tóth and Pernesz (2001):

Chardonnay

An early maturing vine with moderately vigorous growth and a moderate yield, averaging 9–11 t/ha. It is a white wine grape variety, state registered in 1956. It has good frost tolerance, is moderately demanding in terms of exposure and soil, relatively drought-tolerant, susceptible to rotting, has poor fruit setting if the weather is cool during flowering, and requires moderately intensive canopy management. Its quality wine, which has a distinctive aroma and flavour, is delicately acidic, and is excellent for champagne-making.

Sauvignon Blanc

A vigorous mid-season vine with a moderate yield, averaging 8–11 t/ha. It is a white wine grape variety, state registered in 1982. It is sensitive to the exposure, but not to the soil type, it is moderately tolerant of frost and drought, moderately susceptible to rotting, and requires intensive canopy management. The quality wine is heady and full-bodied, with a characteristic, harmonious aroma and flavour.

Királyleányka

This vigorous vine matures moderately late and has a high yield, averaging 10–14 t/ha. It is a white wine grape variety, state registered in 1973. It is moderately demanding in terms of exposure and soil, relatively frost-tolerant, has relatively high demands for humidity and nutrients, is susceptible to rotting and somewhat sensitive to drought, and requires intensive canopy management. Its wine is delicately acidic with a finely balanced flavour and aroma.

Pinot Noir

An early maturing vine with a moderate yield averaging 9–10 t/ha. It is a state registered (1993) red wine grape variety with good frost and drought tolerance. The vines are moderately vigorous, thus requiring relatively little canopy management, but are sensitive to rotting, as emphasised by Vanek (1995). The aromatic wine has a characteristic spicy flavour of an acidic nature, and is relatively rich in pigments.

Kékfrankos

A vigorous, mid-season vine with a moderately high yield, averaging 11–12 t/ha. This red wine grape variety, registered in 1956, has no special exposure or soil requirements and is relatively frost- and drought-resistant; it is not prone to rotting and requires little canopy management. The full-bodied wine is slightly aromatic, with a somewhat acerbic character.

Rootstock: Berlandiere × Riparia T. K. 5BB (Csepregi and Zilai, 1988)

This rootstock produces many branches, which mature well. It has good rooting ability and is compatible with most varieties. It has excellent tolerance of *Phyloxera*, lime and drought. It also has good adaptability to environmental conditions.

Vineyard specifications

The vines were planted in 2000/2001 and were trained using the umbrella system, with a spacing of 3 × 0.8 m, giving a growing area of 2.4 m^2 for each vine. The vines had a trunk height of 100 cm and were pruned to a two-bud spur and a 12-bud cane. The interrows were grassed over in autumn 2002 with a mixture of red fescue and English ryegrass.

The vineyard has a slope of 7–12° from NE to SW, with a corresponding variation in the soil type. The more northerly and the higher-lying parts of the vineyard, planted with Kékfrankos and Pinot Noir, have calcareous, earthy barren soil, while the majority of the area consists of calcareous chernozem soil with forest residues (Chardonnay, Sauvignon Blanc). There is a smaller area of slope deposit soil from forest areas at the bottom of the slope, planted with Királyleányka. The area of each variety affected by drought during the course of the experiment is indicated by an arrow in Figure 1.

The climate of the experimental area is influenced both by the moist air of the Atlantic Ocean, which is cool in summer and mild in winter, and by that of the Mediterranean, which is even more humid and warm in summer and even milder in winter. Very high or low temperatures are rarely experienced in this area, and there is only a short period of frost. The dominant wind direction is N to NW.

Experimental methods

Meteorological data (temperature, rainfall and humidity levels) were recorded and processed using a Luft Opus II instrument (LUFT GmbH, Germany). Soil resistance and moisture data were processed using the 3T System electronic layer indicator developed at the University of Agricultural Sciences in Gödöllő. Drought damage was scored visually, the yield was weighed (kg/vine), the sugar content was determined with a refractometer or using the Rebelein method, and the acid content by means of sodium hydroxide titration (Ásvány et al., 1967).

The experiment was set up in a block design with four replications. The blocks were chosen on the basis of the intensity of drought damage. For each variety two blocks were designated on areas with normal water supplies and two on areas affected by drought. The plot size was 33.6 m^2. Soil water capacity values were recorded on three occasions during July and August. The quantitative and qualitative analysis of the yield was carried out at harvest on September 28th and October 2nd.

Fig. 1. Map of the vineyard in Szomód. Arrows mark the area of each variety affected by drought

Results

Drought-induced damage was recorded on the basis of visual observations and the determination of stress-inducing factors (temperature, rainfall, soil water-holding capacity).

Visual observations

The leaves wilted, yellowed and dropped first in the cluster zone and later over a wider area (Fig. 2). The grapes were small and subject to forced ripening. Fewer reserve nutrients were stored than in 2003, as proved by the maturing of the branches and the low level of frost damage in 2004–2005. Leaf analysis at maturity confirmed these findings. Compared with the same period in 2003, the quantities of N, Fe, Ca, Mg and Zn in the leaves were considerably lower (no foliar fertiliser was applied).

Meteorological data

In 2004 the temperature at flowering was below average, resulting in a delay of 2 weeks in the phenological phases. Flowering began earliest, in Weeks 24–25, for Királyleányka, Chardonnay, Pinot Noir and Sauvignon Blanc, and latest, in Week 27, for Kékfrankos. At this time a large quantity (>90 mm) of rainfall was recorded over the course of a month, leading to the protraction of flowering and poor fertilisation. The longitudinal growth of the clusters took two weeks and the development of the grapes a further three weeks, followed by cluster tightening and ripening. When stress developed due to drought, forced ripening was observed on the areas marked with arrows in Figure 1. The mean temperature from March to October 2004 (15.1°C) did not differ greatly from the 50-year mean (15.3°C), but considerable differences could be observed in the rainfall quantities, which amounted to 326 mm in 2004 compared with a 50-year mean of 425 mm, representing a difference of 24%. The drought was particularly severe in July and August, when rainfall sums were 63% and 39% lower, respectively, than the 50-year mean (Fig. 3).

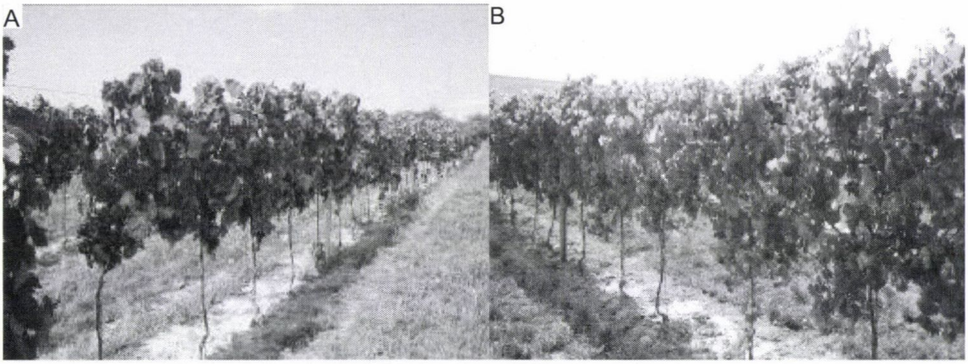

Fig. 2. Vines of the Kékfrankos (A) and Királyleányka (B) varieties during a period of drought

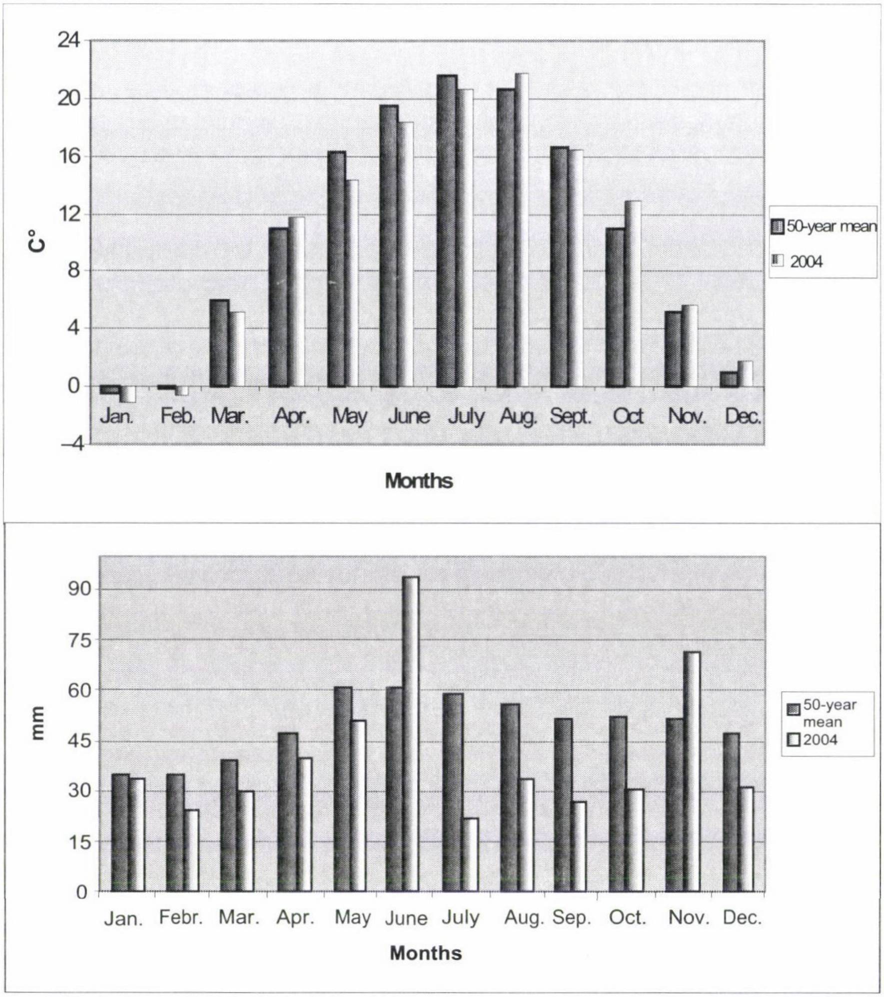

Fig 3. Temperature (A) and rainfall sums (B) in Szomód in 2004 compared with the 50-year mean

Drought damage as a function of soil water-holding capacity

The consideration of non-meteorological factors, such as plant and soil parameters, led to the concept of soil drought, defined as occurring when water deficiency in the root zone becomes the main limiting factor for crop production. Soil drought cannot be determined on the basis of meteorological parameters alone, but requires the knowledge of physical soil properties, including soil water-holding capacity. In most cases soil drought occurs when the moisture reserves of the root zone remain below 30% of field water capacity for a long period. If the moisture level drops to below 15% WHC, irreversible damage to plant development may occur.

When investigating the correlation between the evapotranspiration (ET) of vines and the water-holding capacity of the soil, Kozma (1993) reported minimum values of ET at soil WHC values of around 40% and maximum ET at values above 90 WHC%. Lethal levels of evapotranspiration occurred below the wilting point.

In Szomód values of as much as 53 WHC% were recorded in the 12–20 cm soil layer in 2004, thanks to the plant cover, while at a depth of 40 cm the values ranged from 53% to a hardly detectable 3% (Fig. 4). On the drought-affected area all the values were below the optimum WHC% level. In the case of Kékfrankos and Pinot Noir even "healthy" vines suffered from below-optimum WHC% values. In some cases, at some depths, values below the critical 15% level were recorded. Kékfrankos and Pinot Noir vines were in the most critical state.

Cover plants compete with the vines for both nutrients and water. However, experiments carried out by Rapp and Fox (2004) proved that in the case of advanced drought stress, the interrows should not be tilled. As the result of irrigation and natural rainfall, the root-hair zone of the vines develops below the roots of the cover plant in grassed vineyards, so the vines are able to utilise rainfall quantities as low as 4–6 mm of water. By contrast, in tilled rows this root absorption zone is deeper, so the vines are less able to utilise small quantities of moisture. Thus, despite their competing with the vines, in some cases the cover plants facilitate water uptake.

As seen in Table 1, Királyleányka and Kékfrankos suffered the greatest drought damage in terms of yield losses, with a 66% loss of cluster mass per vine in stressed plots of Királyleányka and a 72% loss in Kékfrankos. Similar changes were observed in the grape berry mass.

Effect of drought on sugar and acid content

The juice of vines grown on drier areas had a lower sugar content and higher acid content than that of vines grown on areas less affected by the drought (Table 2). The greatest reductions in yield quantity and quality were observed for Királyleányka and Kékfrankos, with a 3.3 g/l increase in acid content for the former and a 3.3 MM° (Hungarian must degrees) reduction in the sugar content of the latter as the result of drought stress.

Although the aroma could not be analysed, organoleptic tests indicated that the grape juice had a much poorer, harsher aroma, with a lower extract content and a deficiency in amino acids. The only exceptions were Chardonnay and Sauvignon Blanc, both of which may have been subject to a more moderate state of stress compared to the extreme stress suffered by the other varieties.

Discussion

A study of the relief map and of the enormous differences recorded for soil water-holding capacity and yields raises the question of what could have caused such divergent values within an area of 20.5 ha. As there was no difference in the meteorological parameters, the root stock or the technology, the reason for the differences must be sought in the soil structure and the sensitivity of the varieties.

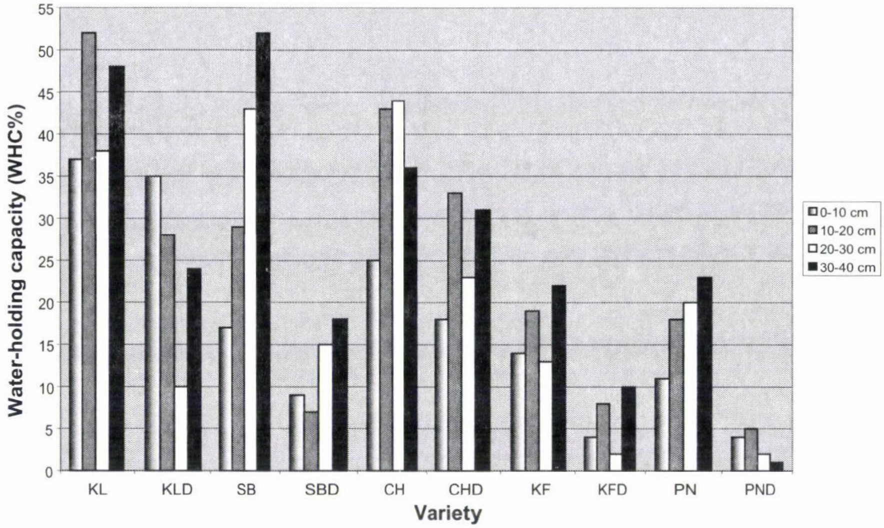

Fig. 4. Values of soil water-holding capacity. The letter D after variety abbreviations (KL: Királyleányka, SB: Savignon Blanc, CH: Chardonnay, KF Kékfrankos, PN: Pinot Noir) indicates plots exposed to drought

Table 1

Changes in the yield per vine as the result of drought stress (Szomód, 2nd October 2004)

Variety	Clusters					Grape berries	
	No.	Mass/vine (g)	Reduction (%)	Mean mass (g)	Reduction (%)	Mass (g)	Reduction (%)
Királyleányka^{+}	38	1500	***66***	39.4	68	0.51	59
Királyleányka^{++}	36	4420		122.6		1.24	
Chardonnay^{+}	25	1680	53	67.2	51	0.61	48
Chardonnay^{++}	26	3580		137.3		1.17	
Sauvignon Blanc^{+}	19	2000	38	105.3	31	0.81	34
Sauvignon Blanc^{++}	21	3200		152.3		1.22	
Pinot Noir^{+}	27	1820	43	67.4	46	0.65	49
Pinot Noir^{++}	26	3220		123.9		1.27	
Kékfrankos^{+}	15	1100	65	73.2	*72*	0.59	***67***
Kékfrankos^{++}	12	3100		258.3		1.77	

$^{+}$: drought-damaged; $^{++}$: healthy

The location of the vines most greatly affected by drought is marked on the relief map by arrows. It is clear that drought caused the most severe problems on higher-lying areas. Soil analyses carried out by the Plant and Soil Protection Service indicate that these areas could be designated as calcareous earthy barren soil, formed on sedimentary rock exposed by erosion. These rocks have poor water management and nutrient-supplying ability. Soil formation, i.e. the long-term manifestation of biological processes leading to humus formation, is inhibited to a great extent by erosion. Due to the shallow topsoil, the soil has unfavourable water management, caused by compaction, poor structure, high lime content and the low content of colloids. For this reason it is prone to drought in dry periods. On calcareous earthy barren soil, the run-off of precipitation water is rapid, with a consequent loss of nutrients. The humus content at a depth of 60 cm is 0.43%. By contrast, the soil of areas less affected by drought was characteristically calcareous chernozem with forest residues. This soil had good water absorption, water storage and water retention, and a humus content of 1.09%.

When studying the effects of drought in 2004, Rapp and Fox (2004) observed the following changes as the result of moderate water deficiency after flowering: the canopy was less dense, the clusters and leaves were well illuminated, the grapes were smaller with a higher sugar content and richer aroma, and ripening accelerated. According to these authors, the growth stage at which the plants are exposed to water deficiency is of great importance. From this point of view four developmental stages can be distinguished in vines after flowering:

I: the first 10–14 days after flowering
II: the cell division phase 14–40 days after flowering
III: the cell growth phase 40–70 days after flowering
IV: further grape berry growth and ripening 70–110 days after flowering.

In phases II and III large quantities of water and nitrogen are required for cell division and growth. If the plants suffer drought stress during this period the phase will be protracted and smaller cell volume will be achieved, leading to smaller clusters. In Szomód in 2004 the plants were subjected to drought during the growth stage of the cells forming the berries, and the effects recorded by Rapp and Fox (2004) were confirmed, i.e. yields dropped to half or a third. The unfavourable effect of drought on the quantity of yield was most clearly perceptible in Királyleányka and Kékfrankos, with yield losses of almost 30%.

Table 2

Effect of drought on the sugar content and acid content of the grape juice (Szomód, 2nd October 2004)

Variety	Sugar content (MM°)	Deviation (MM°)	Acid (g/l)	Deviation (g/l)
Királyleányka drought-damaged	14.8	–1.00	13.5	***3.3***
Királyleányka healthy	15.8		10.2	
Chardonnay drought-damaged	18.4	1.20	10.5	1.5
Chardonnay healthy	17.2		9.0	
Sauvignon Blanc drought-damaged	18.1	–0.10	10.3	0.5
Sauvignon Blanc healthy	18.2		9.8	
Pinot Noir drought-damaged	17.5	–0.30	12.4	0.4
Pinot Noir healthy	17.8		12.0	
Kékfrankos drought-damaged	17.0	***–3.30***	11.2	1.2
Kékfrankos healthy	20.3		10.0	

In the same year Prior (2004) investigated the effect of drought on the quantity and quality of the yield in the Riesling variety, using measurements based not on the water-holding capacity, but on the quantity of soil water available to the vine roots. A soil water potential value of –0.25 MPa was found to retard growth, while photosynthetic activity remained at a normal level, indicating a smaller yield with better quality, as a sufficient quantity of assimilates were available to the clusters. Although the quantity of available water was not recorded, the yield quantity and quality of Chardonnay suggest that a value of 30 WHC% in the root zone represents moderate stress for the plant, causing a rise in the sugar content. This tendency was in agreement with the findings of Prior (2004). In the case of Kékfrankos, however, the extreme drought stress resulted in a 3 MM° reduction in the sugar content.

Porten (2004) reported that there was no substantial change in the acid content of the grape juice from stressed and irrigated (12 L/m^2/week) plants. In the present work, however, the acid content was found to be higher in the juice of all four varieties when the plants suffered from drought than in that of healthy vines.

Conclusions

Based on the values of soil water-holding capacity measured at a depth of 60 cm the varieties could be ranked as seen in Table 3. In the case of Chardonnay the 32 WHC% value recorded in the root absorption zone caused a moderate extent of stress, leading to both a reduction in the yield (53%) and an increase in the sugar content (1.2 MM°).

An extreme level of stress was experienced by the other varieties. Observations and measurements indicated that the varieties Királyleányka and Kékfrankos responded most sensitively to drought in terms of both the quantity and quality of the yield. In Sauvignon Blanc and Pinot Noir, although the yield quantity and to some extent the quality were lower than that of the healthy control, the values of soil water-holding capacity were only 18% and 3%, respectively.

Table 3

Relationship between soil water-holding capacity and the quantity and quality of the yield in drought-stressed vines

Variety	WHC%	Yield loss (%) (100% = healthy vines)	Difference in sugar content compared with healthy vines (MM°)
Chardonnay	32	53	1.2
Királyleányka	24	66	–1.0
Sauvignon Blanc	18	38	–0.1
Kékfrankos	10	65	–3.3
Pinot Noir	3	43	–0.3

MM°: Hungarian must degrees

It can be seen from the results that a soil water-holding capacity of less than 30% at a depth of 60 cm represents extreme drought stress, leading to yield losses of up to 60–65% for high-yielding varieties (e.g. Királyleányka) and a drop of up to 3 MM° (–43 g/L) in the sugar content. At a soil water capacity of 30–40%, however, the mild stress may lead to a lower yield with better quality.

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HISTOLOGICAL ASPECTS OF THE CRYOPRESERVATION OF POTATO

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Cryopreservation appears to be a suitable solution for the maintenance of potato germplasms. The protocol described in this paper can be applied for the vitrification and preservation of meristems. During histo-cytological studies it is possible to observe modifications at the cellular level and to understand the adaptive mechanism to low temperatures. Control potato meristem tissue contained a number of meristematic cells with a gradient of differentiation. After freezing there were a large number of vacuolated cells, some of which exhibited broken cell walls and plasmolysis. The thickening of the cell wall, giving them a sinuous appearance, was observed after freezing and thawing the meristems, with ruptures of the cuticle and epidermal layer.

Key words: apical dome, cryopreservation, meristematic cells, microscopic sections, potato (*Solanum tuberosum* L.)

Introduction

Cryopreservation – storage in liquid nitrogen – is an ideal solution for the long-term maintenance of potato. This procedure seems to be the most suitable for the preservation of rarely used clones, materials and in the case of extremely valuable breeding collections. Research has shown the cryopreservation of apices (Towill and Ros, 1989; Towill, 1990a) to be efficient for germplasm from vegetatively propagated crops. Isolated apices are favoured for the maintenance of clones, since they can be easily regenerated into identical plants. A number of authors have reported the freezing of *Solanum tuberosum* genotypes and other *Solanum* species, and the adaptation of the method to individual varieties (Bajaj, 1978; 1981; Benson et al., 1989; Fabre and Dereuddre, 1990; Grout and Henshaw, 1978; Harding et al., 1991; Henshaw et al., 1985; Schnabel-Preikstas et al., 1992; Towill, 1981a; b; 1983; 1990b). Other studies by Towill (1984) on cryopreservation cover a much broader range of species.

Freezing at –196°C was applied to various tissues of more than 40 species and the level of survival was recorded (Withers, 1980; Morris, 1980). In most cases, cryoprotectants were only added during freezing as supplements. Vitrification is an alternative solution in cryopreservation (Fahy, 1988) and can be applied successfully for the cells, protoplasts and apices of various plant species (Langis et al., 1989; Urigami et al., 1989; Sakai et al., 1990).

In the case of meristems the aim of preserving the whole macroscopic structure is to achieve re-growth without adventitious organogenesis. The survival of potato and date-palm meristems increased when they were cultured on standard media for 1–3 days before the addition of cryoprotectants which promoted their regeneration and the re-induction of their development (Benson et al., 1989; Bagniol et al., 1990). Pre-growth in the presence of cryoprotective materials is a frequently required step (Kartha, 1982; Kartha et al., 1982). There is no general rule for freezing procedures: some species may require rapid freezing, while controlled, slow freezing may be needed for others. Cassava and potato meristems survived even after direct dipping in liquid nitrogen (Bajaj, 1977; Grout and Henshaw, 1978). Towill (1983) reported survival after the slow cooling (0.2–0.3°C/min to –35°C) of meristems originating from *in vitro* potato plantlets. The type of development after thawing depends on the freezing method. Potato meristems show callus development after fast freezing (Benson et al., 1989), while after slow freezing direct re-growth can be observed.

Vitrification techniques have been developed for cell, suspension and protoplast cultures, and for somatic embryos and meristems of various plant species (Urigami et al., 1989; Langis et al., 1989; Langis and Steponkus, 1990; Sakai et al., 1990; Towill, 1990b). The treatment of meristems with vitrification solutions is usually done in two steps. First, the meristems are placed in a low concentration of cryoprotective solution, after which the concentration is increased to the value at which the desired cell contraction occurs. A high concentration of PVS2 lowered the survival rate of potato meristems, as also observed during the vitrification of sweet potato (Towill and Jarett, 1992).

During the present work, a histo-cytological study was carried out at each successive step of the cryopreservation protocol, in order to observe modifications at the cellular level, to understand the mechanisms of adaptation to low temperatures and to identify precisely which steps can be considered as the ‘key steps’ for successful regeneration.

Materials and methods

The experiments were mainly done at the Institut für Pflanzenbau und Pflanzenzüchtung (FAL), Braunschweig, Germany. Tissue cultures of the potato cultivar Adagin maintained for 3 years *in vitro*, were used for cryopreservation. Plants from the *in vitro* collection, maintained with nodal segments containing axillary buds, were placed in test tubes, where inoculations were done every 4–6 weeks on MS (Murashige and Skoog, 1962) basal medium containing 2% (w/v) sucrose and 0.7% (w/v) agar. The cultures were incubated at 25°C under white fluorescent light (approx.

90 μmol m^{-2} s^{-1}) with a 16/8 h photoperiod. Axillary buds containing 3–4 foliar primordia (length about 0.5 mm) and apices (1–2 mm in length) were pre-grown on regeneration medium consisting of MS without hormones and with 2% (w/v) sucrose and 0.8% (w/v) *Difco* agar for 24 hours before cryoprotection.

The vitrification-inducing protocol of Sakai et al. (1990) was modified as follows. The stock solution (100% PVS2), containing 30% (w/v) glycerol, 15% (w/v) ethylene glycol and 15% DMSO (di-methyl-sulphoxide), was diluted with regulator-free MS containing 0.4 M sucrose to form 10, 30, 50 and 70% (w/v) PVS2 concentrations. Pre-treated apices and axillary buds were placed in Petri dishes (50 mm in diameter) containing 1 ml regulator-free MS with 0.4 M sucrose for 5–10 minutes.

The apical and axillary meristems were treated with various concentrations of PVS2 and DMSO. The 10, 30 and 50% solutions of cryoprotective were used for the treatment of meristems at 25°C (room temperature), and the 70 and 100% solutions for vitrification at 0°C (ice). The lower temperature was applied in the case of higher concentrations to minimize toxicity.

After the PVS2 and DMSO treatment 100 selected apical and axillary buds were placed with forceps on 10×20 mm sterile paper sheets previously soaked with 100% PVS2 and cooled to freezing point. The sheets were then folded up and placed in liquid nitrogen (rate of freezing: approx. 4000°C/min) for 1 hour. Thawing was carried out by rapidly transferring the paper sheets into test tubes containing 3 ml MS2 medium (MS in 1.2 M sucrose). The test tubes were incubated in a 30°C water bath for 40 min (rate of thawing: approx. 9000°C/min). Control meristems treated with the vitrification solutions (without fast freezing) were treated similarly and placed in a 30°C water bath without storing in liquid nitrogen.

Treated and control meristems were then put into test tubes containing 8 ml recovery medium, consisting of MS with 0.5 mg/ml benzyl-adenine, 0.1 mg/l indolyl-butyric acid, 3% sugar and 0.7% *Difco* agar, pH 5.8. After treatment the test tubes were kept at room temperature.

Embedding was carried out using Spurr's (1969) ERL embedding solution (ERL-4206+D.E.R.736+NBA+S-1). Infiltration was done in four steps: ERL-acetone in proportions of 1:2, 1:1, 2:1 each for 2 h, followed by ERL overnight at 70°C (polymerisation). The samples were stained with 0.5% toluidine blue and sections were cut with a glass knife.

Micrographs were made with a Carl Zeiss Photomicroscope II: ocular 10×, Neofluar objective 6.3×, 10×, Planapo objectives 25×, 40×, 100× magnifications.

Precisely timed pre-treatments using cryoprotectants with various concentrations and chemical structures are required for satisfactory vitrification, as carried out in the present experiments.

Results

Figure 1 shows the structure of a control sample on a longitudinal section of the apex, indicating the apical dome with two differently aged foliar primordia. The section shows the characteristics of meristematic cells (high nucleoplasmic ratio, dense cytoplasm with small vacuoles). There is a gradient of differentiation from the superficial layers to the central region, with a lower nucleoplasmic ratio and more accentuated vacuolisation. No polysaccharide reserves can be observed.

Figures 2 (apex) and 3 (meristematic cells) demonstrate the state of the structures after freezing. Cellular heterogeneity is a conspicuous phenomenon. Some cells conserved their meristematic characters (in the cellular layers corresponding to the meristem itself). In the underlying zone the cells are more vacuolated and some are damaged, exhibiting broken cell walls and cells where the cytoplasm has contracted away from the cell wall.

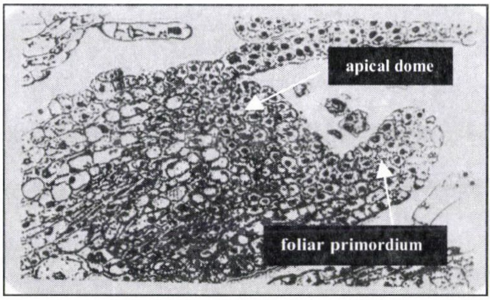

Fig. 1. Structure of the control potato (*Adagin*) sample (longitudinal section of the apex) Magnification: 250×

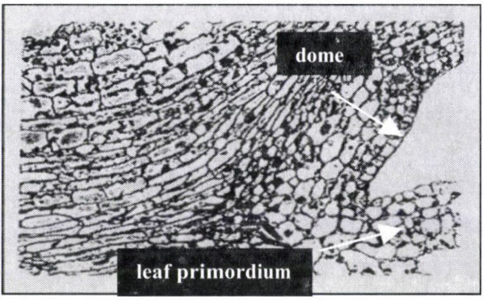

Fig. 2. Histological structure of the apex after freezing. Magnification: 250×

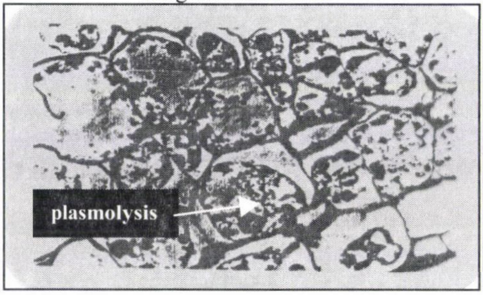

Fig. 3. Histological structure of meristematic cells after freezing. Magnification: 400×

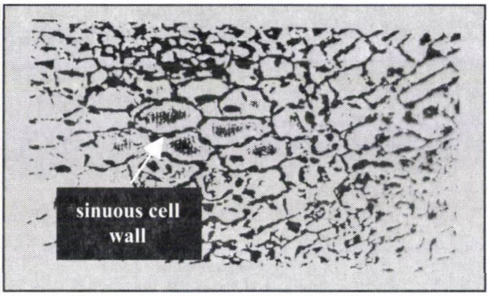

Fig. 4. Histological evaluation of apices during recovery (4–6 days after thawing) Magnification: 400×

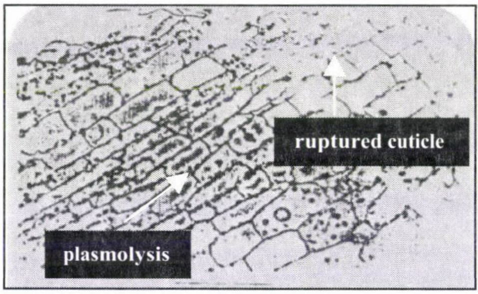

Fig. 5. Phase-contrast micrograph of a frozen and thawed meristem. Magnification: 400×

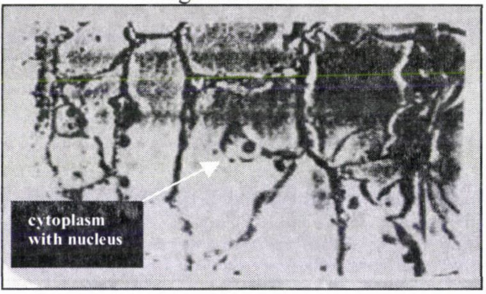

Fig. 6. Section through portions of damaged cells. Magnification: 400×

Figure 4 shows apices during recovery (4–6 days after thawing). The thickening of the cell wall can be observed in some cells in various areas, and from the 6th day onwards both the meristematic and the non-meristematic cells had a sinuous appearance. On the phase-contrast micrograph massive damage to a frozen and thawed meristem can be observed (Fig. 5). A number of cells are ruptured with the total loss of the protoplast; the cuticle and epidermal layer are also ruptured. Damaged cells do not retain the toluidine blue stain.

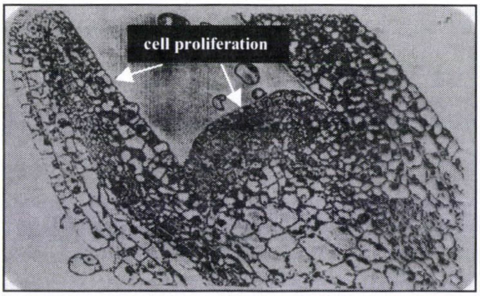

Fig. 7. Histological evaluation of apices during recovery. Magnification: 250×

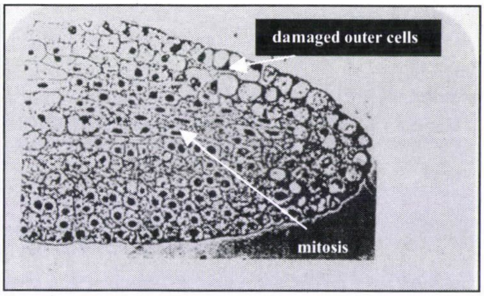

Fig. 8. Apices after thawing and culturing in MSTo medium. Magnification: 250×

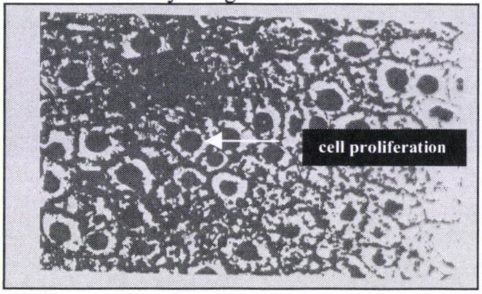

Fig. 9. Richness in starch content in the underlying zone of the meristem. Magnification: 400×

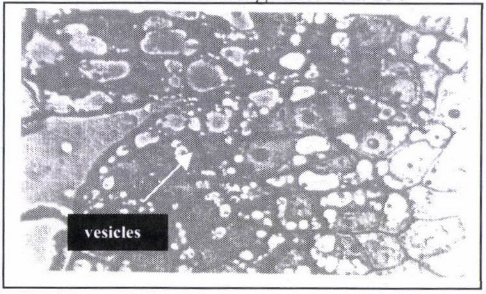

Fig. 10. Section through a leaf primordium and dome an explant that survived freezing and thawing. Magnification: 250×

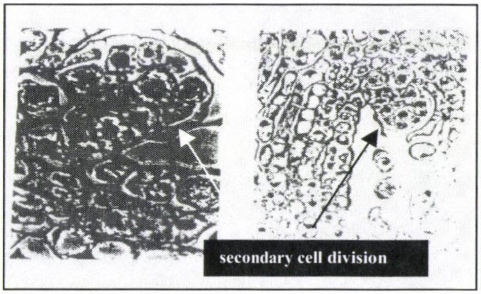

Fig. 11. Histological evaluation after thawing. Magnification: 250×

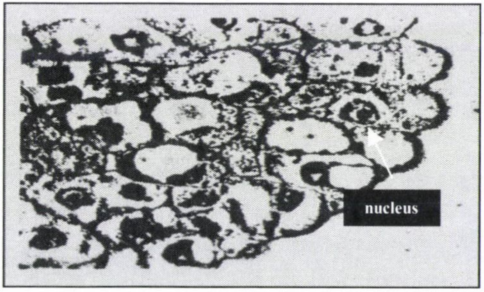

Fig. 12. Histological structure of a non-recovered apex. Magnification: 400×

Figure 6 shows various types of damage in a section through portions of several damaged cells. The plasmalemmas have pulled away from the cell wall and there is evidence of the leakage of cytoplasmic materials through it. In many cases the protoplasts have completely broken down and are separated from the cell wall in places randomly scattered throughout the thawed explant. During recovery many changes can be observed in the apices. Meristematic cells are located around a central zone of damaged cells (cellular zonation).

In the leaf primordia surviving and dividing cells are mainly located in the centre. Figure 7 shows outer cells without a cytoplasm, while the interior cells divide fastest. In the apical dome the first mitosis occurs in the superficial layer. Images of apices after thawing and culturing in MSTo medium (Towill, 1983) can be seen in Figure 8, which illustrates the structure of the apical dome, demonstrating mitosis and cell proliferation.

Figure 9 shows that although the outer cells are damaged, the layer under them is developing rapidly, and a number of cells contain starch grains. The synthesis of starch increased in intensity mostly in cells in the underlying zone, and despite the damage the cells retained a very meristematic appearance.

A section through the leaf primordium and dome of an explant that survived freezing and thawing revealed a number of large 'vesicles' in the damaged cells, together with darkly-staining nuclei and cytoplasm. These 'vesicles' are presumably related to ice-crystal formation, which obscures most of the protoplast detail in Figure 10. Somatic embryogenesis (secondary cell division) can be observed in Figure 11, which shows apices (meristems) with apical domes and leaf primordia. Figure 12 shows the histological structure of a non-recovered apex, in which most cells are highly vacuolated, the majority of the cells have condensed a poorly stained cytoplasm and the nuclei are only just visible.

Discussion

When freezing meristems, the objective is to preserve the whole structure and to induce re-growth without adventitious organogenesis. Surviving cells were mainly located in the superficial layers of the apical dome and in the central region of foliar primordia, where mitosis and cell proliferation could be detected. The method described above was developed for the cryopreservation of old potato varieties stored in a plant genetic resources collection. At present cryopreservation, which basically means keeping the explants in liquid nitrogen, is the only way to guarantee the safe long-term preservation of plant germplasm. In Hungary there is a tradition of *in vitro* storage of potato germplasm (Heszky and Nagy, 1987) and pollen preservation for various cereal species (Barnabás and Rajki, 1976).

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EFFECT OF SOWING DATES ON GENETIC COMPONENTS IN SIX-ROWED BARLEY

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The genetics of yield and related traits was studied in barley (*Hordeum vulgare* L.) by means of 10 × 10 half-diallel progenies (F_1 and F_2) at three sowing dates. An additive-dominance model fitted only for flag leaf area, spike length and 1000-grain weight at different sowing dates. Both additive (D) and dominance components (H_1 and H_2) were significant for all the traits studied, indicating the preponderance of dominance components in controlling the inheritance for these traits. The value of $(H_1/D)^{1/2}$ indicated over-dominance for all the traits except for flag leaf area. Values of 'F' indicated an excess of dominant alleles in the parents for all traits except for flag leaf area. The environmental component 'E' was significant for all traits. The ratio of $H_2/4H_1$ indicated the symmetrical distribution of genes for all the traits studied. The value of h^2/H_2 was less than one for all traits except for spike length, suggesting that a dominant gene was involved in controlling the inheritance of spike length, whereas multiple genes controlled the inheritance of the remaining traits. The heritability estimates were relatively moderate for flag leaf area and 1000-grain weight, but low for all other traits. However, epistatic interactions had an important role in the expression of other traits. Breeding methods such as bi-parental mating in early segregating generations or diallel selective mating may be advantageous to combine important yield component characters for a tangible advance in six-rowed barley.

Key words: six-rowed barley, sowing dates, component analysis, gene effects, quantitative traits, grain yield

Introduction

Barley (*Hordeum vulgare* L.) is the world's fourth most important cereal crop after wheat, maize and rice and is cultivated throughout the temperate and tropical regions of the world. It ranks fourth in acreage and third in terms of crop production. Today, barley is grown on 73 million hectares. The world production of barley is about 160 million tonnes, with the EU being the largest producer, due to the highest yield. Barley is also a very important winter cereal crop in India. By virtue of the lower cost of cultivation, its superior nutritional

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qualities and its multiple uses, barley is promising under less favourable or neglected agricultural conditions such as problematic soils and flood-prone marginal/coastal areas. In India, it is grown on more than 0.66 million hectares, with a production of more than 1.31 million tonnes and a mean yield of 19.84 q ha^{-1} (Anonymous, 2007). In India, barley is grown as a rain-fed or residual moisture crop and is used as human food either for bread making (usually mixed with bread wheat, but also with other cereals or food legumes) or in traditional recipes. The major use of barley grains is in the production of malt, which is used in breweries to make beer, industrial alcohol, whisky, malt syrups, malted milk and vinegar. The spent malt after brewing is used as feed.

Barley cultivation in India is now becoming oriented towards industrial utilization. Though at present only 12–15% of total production is utilized for malting or brewing, it is projected that by 2020 the demand will more than double. There is increasing awareness by industry of the need to improve baking quality, so the breeder is challenged to develop varieties with high yield potential along with high quality for industrial utilization. Improved six-rowed malt genotypes with early maturing and better tillering could further bridge the yield gap and help meet the demand for quality grain for malting purposes. Recently, it has been realized that we have reached a yield plateau, and to achieve a breakthrough in grain yield, systematic and directional breeding should be launched and attempts should be made to find novel genes for the inheritance of desirable traits. The knowledge of gene action, the heritability of the characters and the genetic content of the parents is desirable to develop improved breeding populations.

Considerable emphasis has been placed on improving the yield of barley in the past and will continue in the future. Previous studies on grain yield in barley have shown that it is determined by component traits and is a highly complex attribute (Blum, 1988), and that genes for yield *per se* do not exist (Grafius, 1959). Therefore, knowledge about the magnitude of gene effects of component traits and their expression is of paramount importance in formulating an efficient breeding programme to achieve the desired genetic improvement. Moreover, it is known that the phenotypic expression of quantitatively inherited traits is highly influenced by environmental fluctuations. Genotype × environment interactions lead to bias in the estimates of gene effects for various characters sensitive to environmental modification. Such traits are less amenable to selection, so it is necessary to assess genotype × environment interactions and components of genetic variance for yield to ensure better production and gain under selection. The present study was conducted to analyse the yield performance of progeny from a 10 × 10 diallel under three diverse environments.

Materials and methods

Ten diverse varieties/lines of six-rowed barley (*Hordeum vulgare* L.) selected on the basis of genetic diversity, geographical origin and their suitability for different yield traits, namely RD 2035, RD 2052, RD 2503, RD 2508, RD 2552, RD 2585, RD 387, BL 2, ISBYT 4 and ISBYT 17, were crossed in all possible combinations excluding reciprocals. The 10 parents and their resulting populations of 45 F_1s and 45 F_2s were grown in a randomized complete block design with three replications under early (E_1 – 8th November), normal (E_2 – 23rd November) and late (E_3 – 8th December) sown conditions at Asalpur Research Farm of SKN College of Agriculture, Jobner (26°05' N, 75°201 E, 427 m above sea level) in the Jaipur district of Rajasthan, India. Jobner has a semi-arid climate with an average annual rainfall of about 400 mm, most of which is received between July and early September. Low temperature during the winter season is a characteristic feature of this climatic zone.

Plots of parents and F_1s consisted of two rows 2 m in length, while each plot of F_2s consisted of four rows with a spacing of 30 cm between rows and 10 cm between plants. Ten competitive plants from the parents and F_1s and 30 plants from the F_2 progenies were selected randomly for recording observations on days to heading (75%), plant height (cm), tillers per plant, flag leaf area (cm^2), spike length (cm), number of spikelets per spike, number of grains per spike, 1000-grain weight (g), harvest index (%) and grain yield per plant (g) under each environment separately. All the recommended cultural and management practices were followed to raise a good crop.

The mean of each plot was used for statistical analysis. The data were first subjected to the usual analysis for a randomized complete block design for pooled environments as well as for individual environments (Panse and Sukhatme, 1967). Various components of genetic variance were also computed by the diallel cross method suggested by Hayman (1954).

Results and discussion

The pooled analysis of variance revealed highly significant differences between the genotypes (varieties/lines), indicating that the material used had sufficient genetic diversity (Table 1). Significant differences between the environments also indicated the differential influence of sowing date on character expression. The genotype × environment interaction was found to be significant for all characters, indicating the existence of a non-linear response of the genotypes to the varying environments. This is in conformity with the report that G × E interactions are common in crop plant species (Bhatnagar and Sharma, 1997; 1998). Since a significant genotype × environment interaction was found for all characters, analysis of variance was conducted in individual environments. revealing significant differences between the parents, F_1 and F_2 for all characters in all environments (Table 2). This indicated the presence of diversity in all the material studied. The mean squares for parents vs F_1 and for parents vs F_2 were both significant for all characters in all environments, indicating the presence of heterosis.

The results of covariance–variance regression analysis (b Wr.Vr) revealed the significant deviation of "b" from zero and the non-significant departure of the regression coefficient from unity, indicating the suitability of an additive–dominance model for flag leaf area in both generations and all environments (except F_1E_1), for spike length in both generations in E_3, and for 1000-grain weight in F_2E_2 (Table 3). These characters in the respective generations and environments satisfied the assumptions of diallel analysis. Therefore, component analysis according to Hayman (1954) was carried out only for these characters, whereas epistatic interactions were indicated for all other characters. This shows the importance of testing the genetic material in more than one environment in order to obtain unbiased estimates of various genetic components.

Table 1

Pooled analysis of variance (parents, F_1s and F_2s) showing mean squares for different characters

SOV	d.f.	1	2	3	4	5	6	7	8	9	10
Replication	2	13.15	7.73	0.26	0.19	0.83	13.93	11.05	3.68	3.46	0.58
Environment (E)	2	2879.49**	1625.72**	2.86**	3.43**	0.81*	256.92**	215.73**	972.53**	104.70**	205.21**
Genotype (G)	99	136.21**	561.55**	4.90**	2.26**	2.09**	139.47**	140.07**	53.26**	15.88**	86.28**
Parents (P)	9	165.25**	553.79**	1.94**	5.66**	3.21**	30.62**	33.83**	53.54**	7.92**	15.98**
F_1	44	110.46**	597.07**	4.57**	1.88**	1.35**	145.66**	149.53**	55.72**	15.38**	87.73**
F_2	44	145.88**	527.49**	4.46**	1.77**	1.30**	131.80**	134.29**	49.30**	13.36**	72.31**
P vs F_1	1	625.00**	1090.17**	61.93**	11.68**	59.96**	1320.72**	1072.16**	166.25**	223.16**	13.37**
P vs F_2	1	674.71**	531.56**	66.26**	9.49**	49.11**	887.71**	697.97**	93.77**	102.87**	7.66**
G × E	198	17.56**	15.86**	0.17**	0.42**	0.34**	20.42**	21.85**	4.40**	1.79**	4.26**
P × E	18	7.29	12.45**	0.15*	0.13	0.09	19.52**	17.16**	6.66**	2.24*	2.94**
F_1 × E	88	8.66**	16.49**	0.16**	0.53**	0.36**	21.19**	22.52**	4.11**	1.99*	4.60**
F_2 × E	88	6.33**	16.23**	0.20**	0.38*	0.37**	20.42**	22.86**	3.61**	1.34	3.91**
(P vs F_1)E	2	6.55	11.43	0.05	0.26	0.95*	1.61	4.54	16.80**	9.19**	19.35**
(P vs F_2)E	2	1.00	6.21	0.07	0.52*	0.91*	1.71	3.86	2.77	3.87	17.03**
Error	594	6.28	6.61	0.09	0.17	0.29	8.30	5.87	1.68	1.40	1.05

SOV: Source of variance; 1: Days to heading; 2: Plant height; 3: Tillers per plant, 4: Flag leaf area; 5: Spike length; 6: No. of spikelets per spike; 7: No. of grains per spike, 8: 1000-grain weight, 9: Harvest index, 10: Grain yield per plant; *, **: Significant differences at the 5 and 1 % level, respectively

Estimates of the components of genetic variance revealed that D (additive effects) was highly significant for flag leaf area, spike length and 1000-grain weight (Table 4). Two measures of dominance, H_1 (dominance effect) and H_2 (proportion of dominance due to positive and negative effect of genes), were also highly significant for these traits. Thus, it is suggested that additive and non-additive gene effects were equally important for these characters. However, the relative magnitude of the dominance component was higher than that of the additive component. These findings confirm the results of Bouzerzour and Djakoune (1998), Singh et al. (1999), Vimal and Vishwakarma (1999), Kularia and Sharma (2005), Prakesh and Verma (2006) and Verma et al. (2007).

Estimates of the "F" value, indicating the relative frequency of dominant and recessive alleles in parents, were found to be positive and highly significant for all characters except for flag leaf area in F_2E_1, indicating an excess of dominant alleles (Table 4). The positive but non-significant "F" value for flag leaf area in F_2E_1 gave an indication of an excess of dominant alleles in the parental lines. The environmental component (E) was significant for all characters. The proportion $(H_1/D)^{1/2}$, representing the degree of dominance, was greater than unity for all the characters except flag leaf area in F_2E_3, indicating the existence of over-dominance.

The symmetrical and asymmetrical distributions of genes in the parents were calculated from the ratio $H_2/4H_1$. In order to achieve rapid improvement, it is desirable to have parental material where the genes affecting the characters are symmetrically distributed ($H_2/4H_1 = 0.25$). In the present study, this value was

Table 2
Analysis of variance showing mean squares in individual environments for parents, F_1s and F_2s for different traits

Character	Env.	Replication	Genotype	Parents	F_1	F_2	P vs F_1	P vs F_2	Error
	df	2	99	9	44	44	1	1	198
Days to heading	E_1	3.28	42.92**	38.87**	36.51**	45.58**	284.98**	218.73**	7.01
	E_2	2.90	55.97**	72.85**	48.88**	56.83**	209.60**	216.03**	8.63
	E_3	8.13	54.44**	68.11**	42.40**	56.13**	143.57**	241.55**	9.20
Plant height	E_1	12.02	208.50**	217.08**	215.60**	202.62**	283.93**	157.63**	14.72
	E_2	19.43	218.24**	227.90**	228.95**	202.98**	526.88**	258.98**	26.81
	E_3	29.03	165.51**	133.71**	1.85.50**	154.33**	302.22**	127.35**	21.29
Tillers per plant	E_1	0.31	1.80**	0.75*	1.75**	1.55**	22.27**	25.07**	0.35
	E_2	0.18	1.71**	0.70*	1.40**	1.80**	18.53**	20.92**	0.32
	E_3	0.45	1.73**	0.77*	1.73**	1.50**	21.21**	20.41**	0.33
Flag leaf area	E_1	0.44	1.70**	2.33**	1.74**	1.46**	5.31**	5.91**	0.35
	E_2	0.07	0.74**	2.03**	0.60**	0.54**	4.95**	3.56**	0.24
	E_3	0.02	0.66**	1.56**	0.59**	0.52**	1.94**	1.00*	0.25
Spike length	E_1	0.24	0.74**	1.04**	0.60**	0.53	14.35**	11.65**	0.38
	E_2	0.43	0.98**	1.13*	0.80**	0.81**	16.30**	12.85**	0.51
	E_3	0.61	1.05**	1.23**	0.68**	0.71**	31.21**	26.42**	0.37
No. of spikelets per spike	E_1	4.90	58.82**	32.82**	61.86**	54.41**	391.32**	350.96**	8.94
	E_2	3.22	69.99**	21.03**	75.21**	68.00**	434.62**	259.83**	7.85
	E_3	3.34	51.52**	15.81**	50.98**	50.23**	497.82**	280.64**	8.13
No. of grains per spike	E_1	0.54	58.42**	32.12**	59.51**	57.70**	329.45**	271.99**	6.07
	E_2	2.65	69.68**	18.57**	79.88**	66.23**	297.70**	168.65**	5.48
	E_3	0.96	55.67**	17.47**	55.19**	56.06**	454.08**	265.06**	6.08
1000-grain weight	E_1	1.78	27.23**	27.72**	27.78**	26.82**	39.21**	14.96**	1.83
	E_2	0.18	15.30**	22.14**	14.81**	11.33**	144.11**	51.69**	1.59
	E_3	2.57	19.54**	17.01**	21.34**	18.36**	16.53**	32.65**	1.62
Harvest index	E_1	1.59	6.17**	6.30**	5.37**	5.77**	58.17**	22.99**	1.75
	E_2	2.71	8.06**	3.49**	7.36**	6.58**	144.72**	66.05**	1.37
	E_3	2.57	5.22**	2.61**	6.63**	3.70**	38.65**	21.58**	1.09
Grain yield per plant	E_1	0.58	34.12**	13.49**	35.68**	29.96**	375.41**	227.29**	0.97
	E_2	1.38	34.77**	4.85**	32.26**	29.44**	681.94**	491.85**	1.35
	E_3	1.94	25.91**	3.51**	29.09**	20.92**	318.48**	153.45**	0.82

*, **: Significant differences at the 5 and 1% level, respectively; Env.: Environment

above 0.20 for flag leaf area in F_2E_1, for spike length in E_3 for both generations, and for 1000-grain weight in F_2E_2, indicating the symmetrical distribution of the genes. However, asymmetrical distribution was observed for flag leaf area in various environments.

The ratio $[(4DH_1)^{1/2} + F / (4DH_1)^{1/2} - F]$ indicates the proportion of the total number of dominant and recessive genes among the parents, determining the extent of genetic advance that can be achieved in a particular direction. If the alleles present in the population are predominantly of a recessive nature the response towards selection would be determined by the dominant gene effects. Therefore, the extent of genetic advance likely to be achieved will be limited if dominant alleles are predominant in the population. It is therefore essential to assess the proportion of dominant and recessive genes in the population in order

Table 3

Regression coefficient (b), its standard error, and deviations from zero and unity for different characters in three environments

Character	Env.	F_1				F_2			
		b	SEb	(b–0)/ SEb	(1-b)/ SEb	b	SEb	(b–0)/ SEb	(1–b)/ SEb
Days to heading	E_1	–0.206	0.288	–0.716	4.188**	0.373	0.240	1.553	2.609*
	E_2	0.505	0.328	1.541	1.510	0.033	0.316	0.104	3.062*
	E_3	0.362	0.357	1.012	1.787	0.334	0.225	1.483	2.955*
Plant height	E_1	0.073	0.170	0.430	5.469**	0.065	0.192	0.339	4.880**
	E_2	0.016	0.254	0.632	3.310**	0.304	0.325	0.936	2.140
	E_3	0.313	0.122	2.574*	5.642**	0.245	0.114	2.158	6.638**
Tillers per plant	E_1	0.250	0.067	3.732**	11.190**	0.189	0.091	2.086	8.932**
	E_2	0.394	0.107	3.681**	5.656**	0.267	0.116	2.303*	6.339**
	E_3	0.303	0.110	2.764*	6.368**	0.296	0.140	2.117	5.028**
Flag leaf area	E_1	0.607	0.298	2.038	1.319	0.859	0.285	3.014*	0.496
	E_2	0.680	0.257	2.649*	1.245	0.735	0.210	3.493**	1.259
	E_3	0.677	0.264	2.565*	1.226	0.891	0.239	3.734**	0.458
Spike length	E_1	–0.098	0.321	–0.306	3.424**	–0.054	0.329	–0.165	3.206*
	E_2	–0.228	0.355	–0.643	3.458**	0.054	0.355	0.153	2.665*
	E_3	0.836	0.322	2.596*	0.509	0.879	0.381	2.307*	0.317
No. of spikelets per spike	E_1	0.120	0.230	0.521	3.820**	0.495	0.232	2.135	2.180*
	E_2	0.020	0.172	0.117	5.697**	–0.104	0.150	–0.693	7.348**
	E_3	0.207	0.193	1.073	4.107**	–0.104	0.214	–0.672	5.347**
No. of grains per spike	E_1	0.128	0.228	0.560	3.827**	0.319	0.175	1.821	3.885**
	E_2	0.054	0.157	0.345	6029**.	–0.122	0.135	–0.905	8.311**
	E_3	0.295	0.163	1.803	4.314**	–0.264	0.220	–1.198	5.734**
1000-grain weight	E_1	0.306	0.259	1.181	2.681*	0.051	0.377	0.137	2.517*
	E_2	0.639	0.116	5.508**	3.110*	0.648	0.167	3.884**	2.109
	E_3	0.001	0.210	0.003	4.769**	–0.011	0.279	–0.040	3.628**
Harvest index	E_1	0.326	0.195	1.672	3.453**	0.362	0.094	3.836**	6.750**
	E_2	0.207	0.104	1.993	7.614**	0.135	0.149	0.904	5.797**
	E_3	0.203	0.052	3.928**	15.411**	0.252	0.122	2.057	6.116**
Grain yield per plant	E_1	0.355	0.112	3.169*	5.760**	0.449	0.112	4.005**	4.923**
	E_2	0.178	0.052	3.463**	15.951**	0.110	0.062	1.777	14.357**
	E_3	0.100	0.050	2.001	17.992**	0.134	0.070	1.916	12.413**

*, **: Significant differences at the 5 and 1 % level, respectively; Env.: Environment

to select genotypes for further improvement. In the present investigation, an excess of dominant genes was observed for all the characters studied. The ratio h^2/H_2 indicated the number of effective factors controlling the particular traits. The value of this ratio was less than one for flag leaf area (except F_1E_2) and 1000-grain weight (F_2E_2), indicating that one gene or group of genes was controlling these characters, whereas for spike length the ratio was greater than unity, suggesting a dominant gene was controlling the character.

Heritability estimates (h^2, narrow sense) based on the results obtained in component analysis revealed low to moderate values. Heritability estimates were moderate for flag leaf area in E_3 for both the generations and for 1000-grain weight in F_2E_2, but were low for the rest of the characters. The results clearly indicated that the degree of heritability was greatly influenced by the environment. Similar results were reported by Bouzerzour and Djakoune (1998) and Bhatnagar et al. (2001). Thus, earlier studies and the present study exhibited flexible results on the heritability of the characters studied, particularly in different environments.

Table 4

Estimation of the components of genetic variance for different traits

Components	F_1			F_2				
	Flag leaf area		Spike length	Flag leaf area			Spike length	1000-grain weight
	E_2	E_3	E_3	E_1	E_2	E_3	E_3	E_2
D	0.604*	0.442*	0.292*	0.656*	0.593*	0.438*	0.293*	6.736*
	±0.072	±0.051	±0.074	±0.100	±0.065	±0.036	±0.080	± 0.916
H_1	3.190*	2.278*	5.058*	4.352*	2.543*	1.480*	4.932*	48.210*
	±0.614	±0.436	±0.633	±0.852	±0.556	±0.310	±0.685	±7.797
H_2	2.300*	1.634*	4.328*	4.056*	1.929*	0.986*	4.124*	38.566*
	±0.521	±0.731	±0.538	±0.724	±0.472	±0.264	±0.582	±6.627
F	1.274*	0.786*	0.761*	0.459	1.138*	0.663*	0.780*	10.914*
	±0.332	±0.236	±0.343	±0.462	±0.301	±0.168	±0.371	±4.226
h^2	2.509*	0.911*	16.309*	2.945*	1.760*	0.412*	13.780*	26.368*
	±0.349	±0.248	±0.360	±0.485	±0.316	±0.177	±0.389	±4.436
E	0.073*	0.078*	0.118*	0.121*	0.084*	0.082*	0.118*	0.644*
	±0.022	±0.015	±0.022	±0.030	±0.020	±0.011	±0.024	±0.276
$(H_1/D)^{1/2}$	1.149	1.134	2.081	1.288	1.036	0.919	2.052	1.338
$[\frac{1}{2}(H_1/D)]^{\frac{1}{2}}$	1.625	1.605	2.943	1.821	1.464	1.300	2.901	1.892
$H_2 / 4H_1$	0.180	0.179	0.214	0.233	0.190	0.167	0.209	0.200
$(4DH_1)^{1/2}+F/(4DH_1)^{1/2}-F$	2.696	2.287	1.912	1.314	2.727	2.400	1.960	1.869
h^2/H_2	1.091	0.558	3.769	0.726	0.912	0.418	3.341	0.684
h^2(ns)	14.50	23.50	9.80	17.80	5.80	29.00	12.20	21.00

*: Significant differences at the 5% level

The present study revealed that both additive and non-additive components of genetic variance were involved in governing the inheritance of yield and yield components, although a preponderance of non-additive genetic variance was noted. In such a situation, the most suitable breeding procedure would be one which would utilize the additive genetic variance and at the same time maintain heterozygosity. Therefore, forms of recurrent selection, such as diallel selective mating (Jensen, 1970) or bi-parental mating in early segregating generations (Joshi and Dhawan, 1966) might prove to be an effective approach. This would lead to an elevation of the genetic plateau by accumulating favourable additive genes and simultaneously exploiting the dominance variance. Although it is difficult to produce enough seed in barley by conventional methods, the 'obligate' cross-fertilization system using male sterility, as proposed by Athwal and Borlaug (1967), could bring about large-scale inter-mating between selected genotypes, as envisaged under the recurrent improvement programme.

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EFFECT OF DIFFERENT NUTRIENT LEVELS ON THE RESISTANCE OF SOYBEAN TO *Macrophomina phaseolina* INFECTION IN FIELD EXPERIMENTS

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Field experiments were carried out with soybean [*Glycine max* (L.) Merrill] on a Haplic Chernozem soil. Eleven treatment combinations were applied with increasing rates of fertilizers in three replicates. At full maturity the dry weight production, total biomass production, root weight, yield, shoot NPK concentration and severity of *Macrophomina phaseolina* infection were determined. The highest degree of *Macrophomina* infection was found in the lowest NPK treatment, while the lowest rate of disease was observed for the highest NPK combination. By increasing the NK supply, the degree of infection decreased. At the same fertilizer rates, significantly lower infection was observed at higher phosphorus levels. The dry weight production, total biomass production of the shoots, pod weight and nitrogen concentration of the shoots were negatively correlated with the rate and severity of infection, which was positively correlated with the concentration of phosphorus. No correlation was found between root weight and infection or between potassium concentration and infection.

Key words: *Macrophomina phaseolina*, soybean, resistance

Introduction

Macrophomina phaseolina (Tassi) Goidanich [*Rhizoctonia bataticola* (Taubenhaus) E. J. Butler] can cause serious yield losses in several plant species in Hungary (Békési, 1970; Varga et al., 1997). This polyphagous pathogen infects more than 300 plant species. The disease can be diagnosed on the basis of the symptoms: ash grey spots on the stems and small, black microsclerotia developing in the pith and root tissues (Garamvölgyi et al., 1996). The degree of damage depends on the environmental conditions, habitat, water supply and nutritional level.

The severity of *M. phaseolina* symptoms was increased by higher N rates on sunflower (Zazzerini et al., 1985) and *Phaseolus aureus* (Kartha and Nema, 1969), while Diaz Franco et al. (1989) found that higher N levels reduced the damage. On the other hand, high N levels were found to increase the susceptibility of sunflower to the fungus and high P and K levels to decrease it (Sivaprakasam et al., 1975).

The development of *M. phaseolina* on Indian cotton plants was suppressed in the case of phosphate deficiency (Vasudeva, 1936). It was established that increasing levels of K_2O caused a reduction in the infection rate on jute (De and Chattopadhyay, 1992). The damage caused by *M. phaseolina* to lentil plants could be reduced by increasing K rates (Sinha and Sinha, 2004).

Indra and Grover (1989) reported that N and P significantly increased the severity of *M. phaseolina* infection in mung beans, while K decreased it. In field experiments the rate of fungus infection showed a significant decrease at higher N levels in soybean.

The goal of the present study was to clarify the effect of different levels of NPK supply on the infection of soybean with *Macrophomina phaseolina* in field experiments.

Materials and methods

Field experiments were carried out in 2005 on the soybean [*Glycine max* (L.) Merrill] variety Boróka, bred by the Bóly Agricultural Production and Commercial Share Company. The experimental soil was a chernozem brown forest soil (FAO taxonomy: Haplic Chernozem), the characteristics of which are shown in Table 1. The levels of N, P and K supply were determined according to Egner et al. (1960).

A randomized block experiment was conducted on 33 plots (8.1 m^2 per plot) previously sown to maize. Soybean was sown after fertilizer application on 3 May 2005, with a plant density of 600,000 plants ha^{-1} and a row spacing of 0.45 m.

Besides the unfertilized control, ten fertilizer treatment combinations were applied with increasing rates of fertilizers in 3 replicates (Table 2).

The fertilizer was applied in the form of calcium ammonium nitrate (27%), superphosphate (19%) and potassium chloride (60%).

Table 1
Main characteristics of the experimental soil

Humus	2.25%
AL-P_2O_5 ⁽¹⁾	540 mg kg^{-1}
AL-K_2O ⁽²⁾	277 mg kg^{-1}
$CaCO_3$	2.21%
pH_{H2O}	8.0
Level of N supply	Poor
Level of P supply	Very good
Level of K supply	Good
Water holding capacity	47.6%
pH_{KCl}	7.18

(1) Ammonium lactate (AL)-soluble P_2O_5
(2) Ammonium lactate (AL)-soluble K_2O

Table 2
Fertilizer treatments applied in the experiment

Control	Treatments*									
	1	2	3	4	5	6	7	8	9	10
N0	N0.5	N1	N0.5	N1	N2	N1	N2	N4	N2	N4
P0	P0.5	P0.5	P1	P1	P1	P2	P2	P2	P4	P4
K0	K0.5	K1	K0.5	K1	K2	K1	K2	K4	K2	K4

*Basic units of NPK active agent were the following: N1 = 102 kg ha^{-1}; P1 = 60 kg ha^{-1}; K1 = 126 kg ha^{-1}

Samples each consisting of ten plants were harvested at full maturity and the following parameters were determined: dry matter (DM) production of the shoots (g per sample), total biomass production of the aboveground parts (g per sample), root weight (g per sample), pod weight (g per sample) and NPK concentration of the shoots (%). The natural infection rate and infection index of *M. phaseolina* were determined on plant samples from one row per plot. The infection rate (I%) was calculated as the number of infected plants compared with the total plant number in the sample. The severity of infection (Ii) was determined on a 0 to 5 scale (0: healthy, 5: severe infection).

The plant samples were dehydrated at 60°C. The nitrogen concentration of the shoots was determined after steam distillation according to the Kjeldahl method. The phosphorus concentration was analysed using a spectrophotometer at 430 nm and the potassium concentration with a flame photometer at 760 nm (MÉM NAK, 1978).

The results were statistically analysed (univarient analysis and correlation analysis) using the Microsoft Excel software program package (Microsoft Office XP, 54892-640-4795091-17832). Significant differences were calculated at the 5% probability level.

Results

Increasing NK rates caused a significant decrease in infection with *M. phaseolina* at all P levels except $P_{0.5}$. At the same fertilizer rates, significantly lower infection was found at higher P levels. It was demonstrated that both K and P increased plant disease resistance.

The highest degree of fungus infection was found for the $N_{0.5}P_1K_{0.5}$ treatment, while the lowest infection was observed for the $N_2P_1K_2$ combination (Fig. 1).

Compared to the unfertilized control, both positive and negative differences were found in the rate of *M. phaseolina* infection, depending on the fertilizer rates applied in the experiment. The differences were significant at the P=0.05 level, except for the $N_1P_{0.5}K_1$ treatment. Trends in infection severities were similar to the changes in infection rate.

The results of correlation analysis carried out to obtain more information on the degree of pathogen infection are summarized in Table 3. The critical value of the correlation coefficient (r) was 0.3494 (Fisher and Yates, 1957).

The dry matter production of the shoots, the total biomass production of the aboveground parts and the pod weight showed a close linear correlation with the rate and severity of infection, while the root weight was not correlated with the degree of infection.

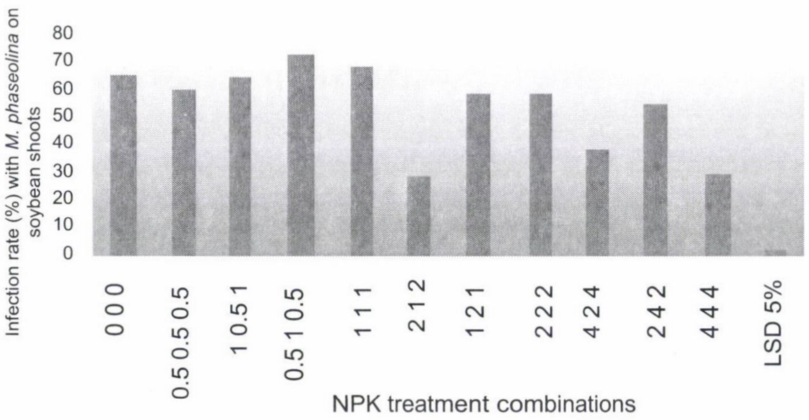

Fig. 1. Rates of infection with *Macrophomina phaseolina* on soybean at different NPK levels

Table 3

Correlation between the infection rates and indexes and the characteristics of soybean samples

	Parameters	R^2	r value	Level of significance (%)
Infection rate	Dry matter production	0.2097	0.4579	1
	Total biomass production of shoots	0.3969	0.6300	0.1
	Root weight (g)	0.0066	0.0812	not significant
	Pod weight (g)	0.4394	0.6629	0.1
	Nitrogen %	0.3156	0.5618	0.1
	Phosphorus %	0.4668	0.6832	0.1
	Potassium %	0.0001	0.0100	not significant
Infection index	Dry matter production	0.1287	0.3587	5
	Total biomass production of shoots	0.2540	0.5040	1
	Root weight (g)	0.0009	0.0300	not significant
	Pod weight (g)	0.2840	0.5329	1
	Nitrogen %	0.3409	0.5839	0.1
	Phosphorus %	0.4213	0.6491	0.1
	Potassium %	0.0050	0.0707	not significant

A close negative correlation was found between the N concentration of the shoots and natural infection with *M. phaseolina*. The concentration of P was correlated with the rate and severity of infection. No significant relationship was observed between the concentration of K and the degree of infection (Fig. 2).

Discussion

The results demonstrated that P and K fertilization increased the resistance of soybean to *Macrophomina phaseolina*.

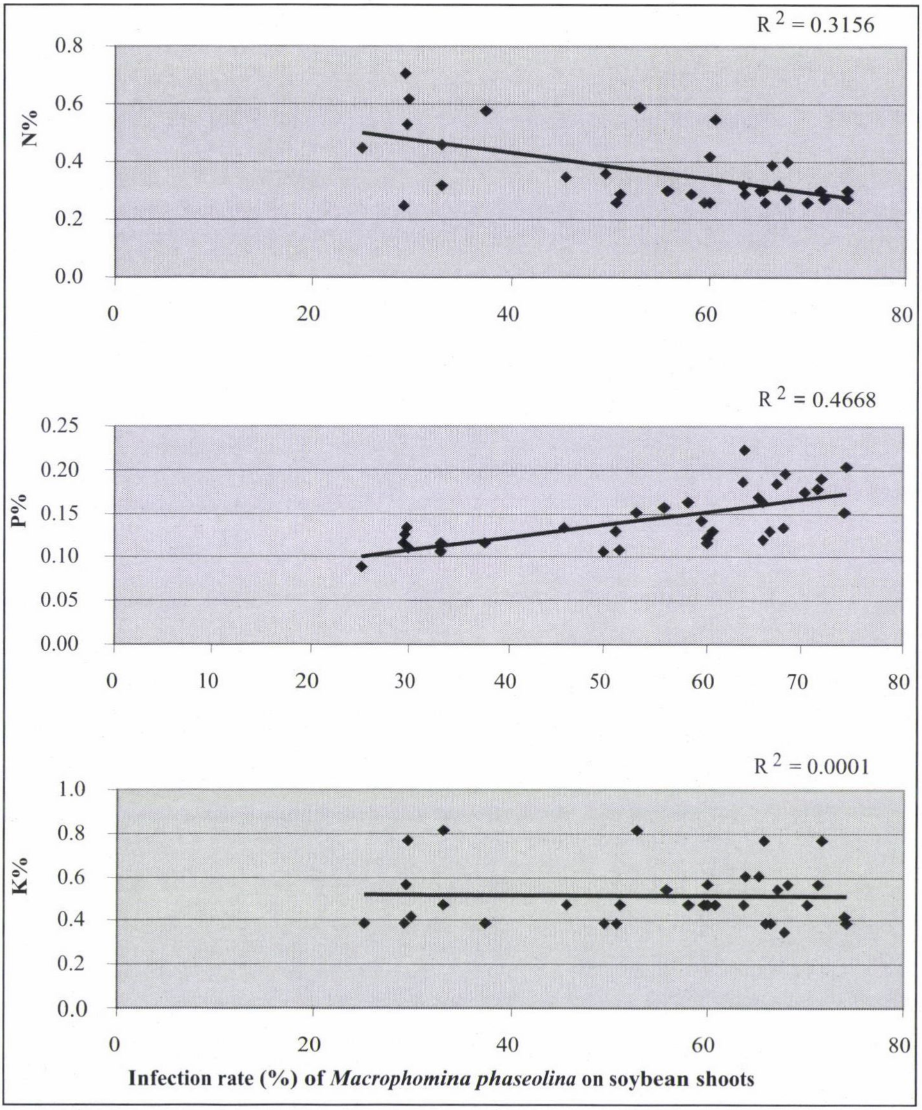

Fig. 2. Rates of natural infection (I%) with *Macrophomina phaseolina* on soybean in relation to the NPK concentration of the shoots

A close negative correlation was found between the rate and severity of pathogen infection and the dry matter production of the shoots, the total biomass production of the aboveground parts and the pod weight of soybean. It was thus concluded that NPK nutrient combinations resulted in higher yield levels, and increased the resistance to *M. phaseolina*.

The concentration of N in the shoots correlated negatively, while the P concentration correlated positively with the infection rates and the severity of symptoms. In these experiments the relationship between fungus infection and the N concentration of the shoots was similar to the results of Diaz et al. (1989). Similar tendencies were reported in cotton plants by Vasudeva (1936) and in mung bean by Indra and Grover (1989). K fertilizers increased plant disease resistance. No significant relationship was found between the K content of shoots and the fungus infection. The K content of the soil decreased the severity of *M. phaseolina* symptoms.

Crop rotation may be helpful, but is not sufficient itself, due to the wide host range of this pathogen.

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SOIL AND PLANT NUTRITIONAL STATUS IN FRUIT ORCHARDS IN SYRIA

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A study was conducted over 15 years in apple, peach, pear, citrus, grapevine and olive orchards in different locations in Syria. The study aimed at monitoring and evaluating the long-term nutrient behaviour in plants and soil in order to suggest measures for nutrient management improvement. Leaf and soil samples were collected in the years 1982, 1987, 1990 and 1997. The soils were characterized by high pH values, high $CaCO_3$ and low to medium organic matter contents. Due to the unfavourable soil conditions and ill-chosen fertilizer use, the nutrient balance in the plant tissues was disturbed. To correct the situation, the application of improved nutrient management practices was suggested.

Key words: nutrient management, soil testing, plant analysis, Syria, fruit orchards, nutrient status

Introduction

The availability of nutrients in the root medium is controlled by many factors (Smith, 1962; Mengel and Kirkby, 1987). The most important of these are the physical characteristics and mineral content of the soil (Page, 1962). Providing fertilizers at the proper time in adequate quantities, taking into consideration the need for balanced, integrated fertilizer application is a key factor in crop production (El-Sayed and Shaaban, 1999; Shaaban et al., 2007). The lower availability of one or more elements leads to growth limitation and low yields (El-Fouly, 1983; 1984). On the other hand, the application of higher fertilizer doses than required may disturb nutrient uptake by the roots as well as their translocation and balance in the plant tissues, thus which decreasing yields.

Agriculture in Syria faces many nutritional problems due to hard soil characteristics. In the early eighties, the Syrian Ministry of Agriculture started cooperation with the National Research Centre Project (Micronutrients and Other Plant Nutrition Problems in Egypt).

The present work is part of the project activities in Syria aimed at the long-term monitoring and evaluation of soil and plant nutritional status in fruit trees in different locations in order to provide proper guidelines for the fertilization process as a part of farm management.

Materials and methods

Apple (*Malus domesticus*), peach (*Prunus persica*), pear (*Pyrus communis*), citrus (*Citrus* sp.), grapevine (*Vitis vinifera*) and olive (*Olea europea*) trees were studied.

Representative soil samples from orchards in different locations were collected from the root absorption area (at 30–60 cm depth) at the beginning of the growth season and before fertilization in the years 1982, 1987, 1990 and 1997.

The samples were sun-dried, ground and passed through a 2 mm sieve. Mechanical analysis was carried out using the hydrometer method (Bouyoucos, 1954), electric conductivity (E.C.) and pH were determined from a 1:2.5 soil : water extract (Jackson, 1973), calcium carbonate ($CaCO_3$) content was studied using the calcimeter method (Black, 1965) and organic matter (O.M.) content with the potassium dichromate method (Walkley and Black, 1934). Phosphorus (P) was extracted using sodium bicarbonate (Olsen et al., 1954), and potassium (K) and magnesium (Mg) using the ammonium acetate method (Chapman and Pratt, 1978). Iron (Fe), manganese (Mn), zinc (Zn) and copper (Cu) were extracted using the DTPA method (Lindsay and Norvell, 1978).

Apple, pear and peach leaf samples were taken in June/July, citrus in Sep./Oct., grapes in May/June and olive in April/May (Robinson, 1988). The age of the trees ranged from 5 to 20 years old.

The collected leaves were washed consecutively using tap water, 0.01 N HCl and bidistilled water, dried at 70°C for 24 h and ground. The plant material was dry-ashed in a muffle furnace at 550°C for 6 h. The ash was digested in 3.0 N HNO_3 and the residue was then suspended in 0.3 N HCl (Chapman and Pratt, 1978).

Phosphorus was spectrophotometrically determined using the molybdate-vanadate method according to Jackson (1973). Potassium was measured using a Lang – M8D Flame photometer. Mg, Fe, Mn, Zn and Cu were determined using a PMQ3 atomic absorption spectrophotometer.

Soil nutrient status was evaluated according to the sufficient concentrations reported by Ankerman and Large (1974) and leaf nutrient status according to Robinson (1988).

The data were analysed using the NCSS computer programme, version 5.3 (Hintze, 1990) to calculate means, standard deviations (SD) and correlation coefficients (r).

Results and discussion

Soil analysis

The soils were characterized by high pH values and $CaCO_3$ contents (Table 1), which may lead to the low availability of micronutrients and phosphorus. However, the pH values tended to decrease in apple and citrus soils, while the $CaCO_3$ content became lower in apple, peach, citrus and grapevine soils in recent years. This might be attributed to an increase in soil organic matter content (Mengel and Kirkby, 1987). No problems with soluble salts are foreseen, as they showed very low E.C. values. The soils were of loamy, sandy clay and calcareous clay texture.

Table 1

Soil physical characteristics evaluated according to Ankerman and Large (1974)

Character	Year	Apple	Peach	Pear	Citrus	Grapevine	Olive
pH	1982	8.1 H	7.5 H	7.5 H	8.0 H	7.2 M	7.2 M
	1987	7.5 H	7.6 H	8.1 H	7.8 H	7.8 H	7.5 H
	1990	7.4 H	7.8 H	7.9 H	8.0 H	8.5 H	7.5 H
	1997	7.7 H	8.4 H	8.3 H	7.4 H	8.3 H	7.4 H
	Mean ± SD	7.76 ± 0.3	7.83 ± 0.17	7.95 ± 0.34	7.8 ± 0.28	7.95 ± 0.58	7.40 ± 0.14
	r	–0.46	–0.21	0.87	–0.82	0.81	0.48
E.C. (dS/m)	1982	0.71 vL	1.05 vL	1.05 vL	1.05 vL	0.72 vL	0.30 vL
	1987	0.23 vL	0.52 vL	0.72 vL	1.0 vL	0.59 vL	0.27 vL
	1990	0.43 vL	0.52 vL	0.78 vL	0.82 vL	0.46 vL	0.55 vL
	1997	0.58 vL	0.51 vL	0.80 vL	0.77 vL	0.35 vL	0.80 vL
	Mean ± SD	0.48 ± 0.2	0.65 ± 0.26	0.83 ± 0.14	0. 91 ± 0.13	0.53 ± 0.16	0.48 ± 0.24
	r	–0.09	–0.75	–0.58	–0.92	–0.98	0.92
O.M. (%)	1982	2.89 M	2.22 M	2.90 M	2.18 M	1.22 L	2.80 M
	1987	1.06 L	1.24 L	2.80 M	2.78 M	3.00 M	3.10 H
	1990	1.56 L	2.24 L	3.10 H	2.68 M	2.30 M	2.40 M
	1997	2.18 M	2.56 M	3.25 H	2.53 M	1.52 L	1.87 L
	Mean ± SD	1.29 ± 0.79	2.06 ± 0.57	3.01 ± 0.20	2.54 ± 0.26	2.01 ± 0.8	2.52 ± 0.57
	r	–0.22	0.43	0.84	0.42	–0.05	–0.85
$CaCO_3$ (%)	1982	60.0 vH	26.5 H	48.4 vH	29.5 H	12.0 H	16.9 H
	1987	62.0 vH	23.5 H	49.4 vH	28.0 H	12.5 H	16.7 H
	1990	58.5 vH	23.0 H	49.0 vH	22.0 H	10.5 H	15.5 H
	1997	59.5 vH	23.9 H	50.2 vH	21.4 H	8.30 H	19.9 H
	Mean ± SD	60.0 ± 1.47	24.2 ± 1.56	49.2 ± 0.75	25.2 ± 4.10	10.8 ± 1.80	17.2 ± 1.80
	r	–0.34	–0.62	0.91	–0.89	–0.90	0.65

SD = standard deviation, H = high, vH = very high, M = moderate, L = low, vL = very low, r = correlation coefficient

Soil nutritional status (Table 2)

Macronutrients

Phosphorus contents were very high in general, but they tended to be high or moderate in the last year of the study. Potassium values were found to decrease. In 1997, medium and very low K contents were detected in peach, pear, citrus and olive soils. Inadequate concentrations of elements in the soil may disturb the nutrient balance and consequently the uptake and distribution of other nutrients in the plant tissues (El-Fouly and Shaaban, 1999).

Micronutrients

The available iron content remained very low or low throughout the study period. The available manganese content ranged from very low to high in the last two years of the study. Soil zinc concentrations tended to decrease. Copper concentrations exhibited high or very high values, possibly as a result of pesticide applications. The soil application of micronutrients is not generally recommended under such soil conditions (high pH and high $CaCO_3$ content)

(Alexander, 1986). To counteract the low availability of soil micronutrients, chelated compounds should be used as foliar application (El-Fouly et al., 1997; Shaaban et al., 2007).

Table 2

Available macro- and micronutrient contents in the soil, evaluated according to Ankerman and Large (1974)

Element	Year	Apple	Peach	Pear	Citrus	Grapevine	Olive
Phosphorus (mg/100 g soil)	1982	2.30 M	6.65 vH	11.4 vH	8.70 vH	1.90 M	6.50 vH
	1987	6.00 vH	9.50 vH	12.4 vH	10.2 vH	1.13 L	8.20 vH
	1990	10.5 vH	5.18 vH	6.25 vH	4.85 vH	2.22 M	6.16 vH
	1997	11.9 vH	3.16 H	2.35 M	2.59 M	3.27 H	2.82 H
	Mean ± SD	7.67 ± 4.37	6.12 ± 2.66	8.10 ± 4.68	6.58 ± 3.48	2.13 ± 0.88	2.92 ± 2.28
	r	0.94	–0.69	–0.90	–0.84	0.76	–0.78
Potassium (mg/100 g soil)	1982	28.0 M	28.5 M	53.3 vH	13.2 L	25.7 M	12.2 L
	1987	17.0 L	16.7 L	50.9 vH	13.7 L	93.9 vH	14.9 L
	1990	21.6 M	22.4 M	32.4 H	11.5 vL	83.5 vH	10.1 vL
	1997	36.2 H	28.6 M	11.4 vL	9.62 vL	52.7 vH	5.02 vL
	Mean ± SD	25.7 ± 8.30	24.05 ± 5.69	37.0 ± 19.45	12.0 ± 1.84	83.9 ± 30.9	10.5 ± 4.18
	r	0.51	0.71	–0.95	–0.90	0.23	–0.82
Magnesium (mg/100 g soil)	1982	18.2 L	22.0 L	16.7 L	23.5 L	18.9 L	14.0 L
	1987	33.1 M	35.1 M	25.8 M	21.8 L	36.5 M	16.5 L
	1990	44.8 M	56.8 M	54.2 M	30.5 M	50.8 M	22.9 L
	1997	56.3 M	76.7 M	58.5 M	38.0 M	51.0 M	30.1 M
	Mean ± SD	38.1 ± 16.3	47.6 ± 24.1	38.8 ± 20.6	28.4 ± 7.40	39.3 ± 15.2	20.8 ± 7.20
	r	0.98	0.98	0.91	0.90	0.88	0.97
Iron (mg/kg soil)	1982	2.80 vL	3.30 vL	9.10 L	6.30 L	4.37 vL	2.80 vL
	1987	6.00 L	4.37 vL	9.80 L	6.40 L	5.50 L	4.61 vL
	1990	8.72 L	5.80 L	8.60 L	6.50 L	6.90 L	5.00 L
	1997	13.4 M	3.10 vL	5.65 L	2.85 vL	5.30 L	5.40 L
	Mean ± SD	7.73 ± 4.48	4.14 ± 1.23	8.28 ± 1.82	5.51 ± 1.77	5.51 ± 1.04	4.45 ± 1.14
	r	0.99	–0.05	–0.85	–0.82	0.39	0.85
Manganese (mg/kg soil)	1982	3.60 vL	4.10 vL	4.20 vL	6.30 L	8.95 M	7.40 L
	1987	4.57 vL	8.50 M	12.1 M	10.5 M	8.50 M	18.1 H
	1990	16.5 H	14.5 H	10.2 M	19.2 H	20.2 H	23.5 H
	1997	29.6 H	21.0 H	8.26 M	28.0 H	33.0 H	26.1 H
	Mean ± SD	13.5 ± 12.2	12.0 ± 7.30	8.69 ± 3.37	16.0 ± 9.60	17.6 ± 11.5	18.7 ± 8.20
	r	0.95	0.98	0.35	0.98	0.93	0.92
Zinc (mg/kg soil)	1982	3.40 H	3.42 H	3.30 H	1.90 M	4.80 H	2.80 M
	1987	2.28 M	4.80 H	3.20 H	1.70 M	4.50 H	2.28 M
	1990	2.10 M	2.15 M	2.80 M	1.64 M	3.50 H	1.12 L
	1997	2.05 M	1.12 L	1.00 L	1.14 L	1.22 L	0.92 L
	Mean ± SD	2.45 ± 0.63	2.87 ± 1.59	2.57 ± 1.07	1.59 ± 0.32	3.50 ± 1.62	1.78 ± 0.90
	r	–0.82	–0.74	–0.92	–0.97	–0.96	–0.11
Copper (mg/kg soil)	1982	4.25 vH	4.20 vH	2.90 vH	3.20 vH	5.20 vH	3.90 vH
	1987	3.12 vH	2.80 vH	2.50 vH	3.40 vH	4.84 vH	3.86 vH
	1990	4.20 vH	2.14 H	2.25 H	2.80 vH	4.20 vH	2.20 H
	1997	5.27 vH	1.86 H	2.25 H	1.41 H	3.20 vH	1.07 M
	Mean ± SD	4.21 ± 0.87	2.75 ± 1.04	2.47 ± 0.30	2.70 ± 0.89	4.36 ± 0.87	2.75 ± 1.37
	r	0.62	–0.91	–0.87	–0.89	–0.98	–0.93

SD = standard deviation, H = high, vH = very high, M = moderate, L = low, vL = very low, r = correlation coefficient

Plant nutritional status

Macronutrients

Figure 1 shows the leaf macronutrient concentrations in the studied crops as compared to the recommended sufficient values of Robinson (1988). Nitrogen status was improved in the two last samplings as a result of recommendations from the Syrian Soil Directorates for all the studied crops, reaching high levels in some years. Phosphorus concentrations were always in the sufficient range, reflecting its adequacy in the soil. Despite its low levels in the soil, the potassium concentration in the leaves was always in the sufficient range, and even above the upper level of sufficiency in some cases. Magnesium concentrations were generally in the sufficient range except in olive trees.

Micronutrients

The micronutrient concentrations in the leaves of apple, peach, pear, citrus, grapevine and olive compared to the sufficient ranges recommended by Robinson (1988) are shown in Fig. 2. As a result of micronutrient foliar application, the iron and zinc concentrations clearly improved over the years, reaching the sufficient level. The manganese concentrations were always in the sufficient range throughout the study period. The copper concentrations were always in the sufficient range or even higher in some cases. Thus, elaborating fertilizer recommendations according to soil testing and leaf analysis has succeeded in increasing the nutrient levels in the plants.

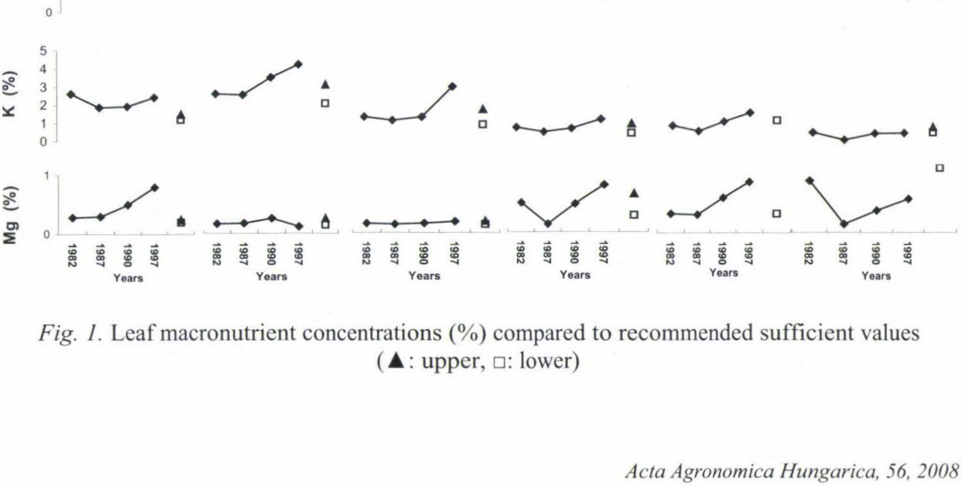

Fig. 1. Leaf macronutrient concentrations (%) compared to recommended sufficient values (▲: upper, □: lower)

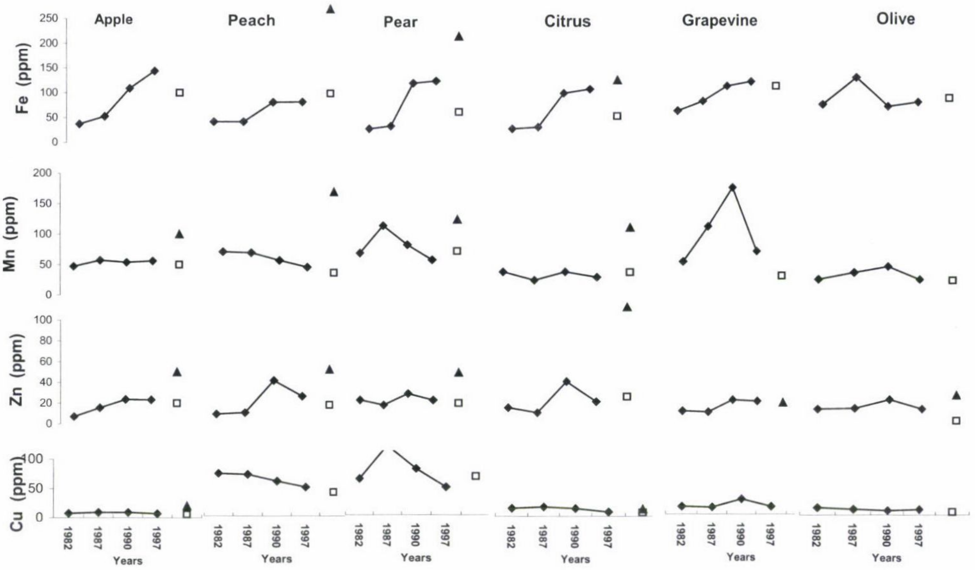

Fig. 2. Leaf micronutrient concentrations (ppm) compared to recommended sufficient values (▲: upper, □: lower)

Nutrient balance

Table 3 shows calculations of the leaf nutrient concentration ratios actually present in the compared to the sufficient values recommended in the literature. The data revealed that some nutrient ratios in the leaves were disturbed. Imbalances in the N/P, N/K and N/Fe ratios were found, which might be due to the high N doses used. On the other hand, soil potassium deficiency may have disturbed the magnesium uptake, consequently disturbing the K/Mg ratio in the leaves. As micronutrients were added as foliar application, as recommended, the N/Fe, P/Mn, P/Zn and Fe/Zn ratios were close to the recommended sufficient values. Nutrient imbalance within the plant tissues generally caused a disturbance in the physiological and biochemical processes, leading to yield decreases (Fawzi et al., 1996; Shaaban and Abou El-Nour, 1996; El- Fouly and Shaaban, 1999; Shaaban et al., 2007). Accordingly, nutrient management should be implemented in an integrated and balanced manner.

Conclusions

From this work, it could be concluded that:

– The fruit trees studied in Syria are suffering from unfavourable soil conditions. The addition of soil conditioners and acidic fertilizers could improve the soil nutritional status to some extent.

– Fertilizer recommendations based on soil testing and leaf analysis led to an improvement in the nutrient concentrations in the leaves.

– Nitrogen and potassium fertilizers should be optimally applied at the proper time.

– Micronutrients in chelated forms should be continuously applied to the leaves in order to meet crop requirements.

Table 3
Calculated nutrient ratios in the leaves of the studied crops compared to the sufficient ranges cited from literature

Crop	Year	N/P	N/K	K/Mg	N/Fe	P/Mn	P/Zn	Fe/Zn
Apple	1982	9.45	0.79	9.35	554.6	46.8	314.2	4.96
	1987	7.00	0.82	6.23	229.7	38.6	141.0	5.68
	1990	10.7	1.10	3.88	196.8	37.0	84.7	12.2
	1997	30.1	0.99	3.00	148.4	14.2	34.3	15.0
	Suff. values	12–13.3	1.60	4.6–5.3	200	26–30	40–75	20–25
Peach	1982	13.0	0.83	5.43	4.14.3	22.4	130.0	4.08
	1987	11.0	0.76	5.46	376.0	24.4	127.8	3.75
	1990	18.9	3.48	2.34	559.0	42.6	61.7	2.09
	1997	30.7	0.83	6.11	396.2	22.5	40.14	3.11
	Suff. values	14–21.4	1.16–1.5	3.75–6.66	140–300	15.6–35	50–70	5.0
Pear	1982	8.56	0.85	5.92	472.4	28.6	69.5	1.26
	1987	9.28	0.92	5.03	379.0	13.0	76.9	1.88
	1990	16.9	1.70	2.83	239.6	22.2	54.4	3.84
	1997	34.4	0.96	3.72	264.7	20.9	39.3	5.11
	Suff. values	13.5–14.6	1.35–1.9	4.0	135–383	16.6–22.3	40–70	3.0–4.0
Citrus	1982	9.50	1.33	2.17	348.1	54.9	25.0	2.44
	1987	9.75	1.51	6.41	383.2	100.0	103.4	3.56
	1990	17.8	3.00	2.15	290.2	62.7	42.5	2.61
	1997	41.6	2.58	1.95	357.1	54.2	43.4	5.07
	Suff. values	16.2–20	2.16–3.42	2.0–2.69	216–400	16–48	16–48	1.2–2.4
Grapevine	1982	9.62	1.28	4.00	273.0	32.8	82.9	2.92
	1987	9.22	1.88	3.14	226.7	16.6	98.9	4.02
	1990	11.9	2.97	5.76	429.0	20.6	127.6	3.54
	1997	33.1	1.88	2.46	342.8	91.7	58.8	3.97
	Suff. values	12.5–20	> 2.6	> 5.0	> 400	> 80	> 76.9	> 3.84
Olive	1982	6.27	1.38	1.25	156.6	97.7	113.9	4.56
	1987	7.81	2.25	4.61	98.1	178.5	89.8	6.94
	1990	17.3	2.73	2.79	309.5	34.8	56.3	3.15
	1997	28.7	2.95	1.86	310.6	45.6	51.8	4.78
	Suff. values	6.6–15	> 1.5	> 1.0	> 150	> 50	100	> 2.5

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Short communication

HIGH FORAGE YIELD AND QUALITY OF SUDAN GRASS (*Sorghum bicolor* L.) AND PEARL MILLET (*Pennisetum glaucum* L.) CULTIVATED IN CALCAREOUS SOILS

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Sudan grass and pearl millet are major warm season dryland crops, primarily grown for grain production and used as a major source of dietary energy. Both crops are highly water use efficient and belong to the C_4 group of species with high photosynthetic efficiency and dry matter accumulation rates. Both sudan grass and pearl millet have good forage quality, with an adequate crude protein content; that of pearl millet (8.7%) is higher than that of sorghum (6.0%). Therefore, the present investigation was conducted in the Western Delta Region at El-Naubaria, 40 km south of Alexandria, over two successive seasons to evaluate the forage yield and quality of sudan grass and pearl millet. Field experiments were established on calcareous soils, with five fertilization treatments. The results indicate that pearl millet surpassed sudan grass in fresh (6.56 t/ha) and dry yield (2.91 t/ha), which was 8.89% and 5.26% more, respectively, than for sudan grass. As regards the forage quality, pearl millet had good digestibility and was lower in fibre than sudan grass.

Key words: sudan grass, pearl millet, forage quality, fertilization

Introduction

In view of global climate change, rising temperatures, increasing water shortages and salinity, and deteriorating natural resources, there is a growing demand for alternative forage resources of high quality to meet the increased demand for animal production (meat and milk).

Sudan grass and pearl millet evolved as leafy structures, destined to be forage crops rather than grain crops. They have high water use efficiency (WUE), reported to be, respectively, 27% and 93% greater than maize, when grown under limited environments (Singh and Singh, 1995). In addition, they are well adapted to dry areas, having a high degree of drought tolerance, and also have a high degree of adaptation to problem soils and degraded lands (Peacock et al., 1993).

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Both crops are C_4 species with high photosynthetic potential and dry matter production ability. In an experiment conducted under severe water stress, sorghum and pearl millet gave 4.1 t/ha dry matter, i.e. 27% more than maize (Singh and Singh, 1995). In this respect the study of Kim et al., (2000) in Korea showed that sorghum gave 7% more dry matter and pearl millet 32% more dry matter in a single cut than maize, which produced a dry matter yield of 15.5 t/ha.

Many cropping systems have been developed to enhance the productivity of tropical and even subtropical crops. In these systems, the use of organic matter, especially in the form of manure and crop residues, plays a prominent role (Fischler et al., 1999). Duraisami and Mani (2002) reported that the use of composted coirpith provided a better nutritional environment in groundnut and also enhanced the residual effect for the succeeding crop. Composted coirpith application along with 75% or 100% of the recommended dose of chemical fertilizers resulted in a higher yield of both crops. The nutritional uptake increased parallel with the N levels. This in turn alleviated the stress of poor groundwater and nutrient retention, while improving soil microbial activity and thus providing a more conducive environment for crop growth.

Materials and methods

Two field experiments were conducted during the summer seasons of 2002 and 2003 in Nobaria District, El-Behira Governorate, aimed at developing cultivation practices involving various fertilization treatments to improve the yield and quality of two forage crops. Before commencing the experiment, samples were taken from each layer of the soil profile and from the groundwater for physical and chemical analyses by the methods described by Cottenie et al. (1982) and Burt (2004). The results are presented in Tables 1 and 2.

A split-plot design with four replications was laid out involving ten treatments, which were combinations of two forage crops (sudan grass and pearl millet) and five fertilization treatments: organic manure; chemical fertilizers (application of N at the rate of 215 kg/ha); organic manure + biofertilizers (seeds mixed with arabic gum as adhesive material and specific multi-strain bacteria four hours before sowing); chemical fertilizers + biofertilizers; organic manure + biofertilizers + chemical fertilizers.

Seeds of a local cultivar of sudan grass and pearl millet cv. Shandaweal–1 were sown on 10th and 15th May in the first and second seasons, respectively. Calcium superphosphate (15.5% P_2O_5) at a rate of 350 kg/ha and 150 kg/ha potassium as potassium sulphate (48% K_2O) were applied during soil preparation. Irrigation took place every 10–14 days by flood irrigation methods. During the growth period nitrogen fertilizer was applied as ammonium sulphate (20.6% N) at a rate of 215 kg/ha in three equal doses. Two cuts were taken at 50 and 100 days from sowing.

Forage quality was determined in the second cut. Samples were taken from each treatment to determine the crude protein content from the nitrogen content (using the micro-kjeldahl method) and to calculate the protein content, fibre %, ash %, fat %, soluble carbohydrate content and digestibility %.

The data obtained for each year were subjected to statistical analysis according to Gomez and Gomez (1984). The same trends were observed, so combined analysis of the two-year experiment was done according to Gomez and Gomez (1984). The means were compared by the least significant difference (LSD) method at the 5% level.

Table 1
Chemical properties of the soil of the studied profile

Depth	SP (%)	pH	EC (dS/m)	Soluble cations (meq/l)				Soluble anions (meq/l)				CaCO3 (%)
				Ca^{2+}	Mg^{2+}	Na^+	K^+	CO_3^{2-}	HCO_3^-	Cl^-	SO_4^{2-}	
0–25	53.3	8.30	4.34	11.1	7.9	22.4	2.0	0.0	1.2	23.5	18.7	18.46
25–70	48.3	8.20	3.63	10.5	7.2	16.9	1.7	0.0	1.0	22.9	12.4	30.77
70–120	50.0	8.17	2.50	8.1	6.0	9.7	1.2	0.0	0.9	14.3	9.8	34.62

pH was measured in a soil suspension (1:25); EC was measured in extracted soil paste

Table 2
Chemical properties of the groundwater used for irrigation

pH	EC dS/m	Soluble cations (meq/l)				Soluble anions (meq/l)			
		Ca^{2+}	Mg^{2+}	Na^+	K^+	CO_3^{2-}	HCO_3^-	Cl^-	SO_4^{2-}
7.80	5.64	11.5	8.5	33.5	2.8	0.0	1.4	38.2	16.8

Results and discussion

Forage yield

Sorghum and pearl millet have evolved as leafy structures, destined to be forage crops rather than grain crops. The results in Table 3 clearly reveal significant differences between the two crops in the different fertilization treatments. The data showed that the application of organic manure in the presence of chemical fertilizers and treating the seed before sowing with specific bacteria had the highest effect on the fresh and dry yields of both crops. The increment in the fresh forage yield of sudan grass was 12.54% and 6.86%, respectively, compared with the application of organic manure and chemical fertilizers. In addition, the increment in the fresh forage yield of millet was higher than in sudan grass when chemical fertilizers were applied in the presence of organic manure and biofertilizers. The forage yields were increased by 27.77% and 21.62% compared with the application of organic manure and chemical fertilizers, respectively. The interaction between crops and fertilization treatments also showed the same trend. The data in general supported the findings of Fischler et al. (1999), Kim et al. (2000) and Duraisami and Mani (2002).

Forage quality

The data in Table 4 indicated that pearl millet had good quality forage with high crude protein content and digestibility and with low fibre and lignin percentages. The crude protein content of pearl millet (8.6%) was 19.44% higher than that of sudan grass. The results also showed that the digestibility percentage was greater in the first cut compared with the second one, probably due to the higher fibre content in the second cut. It can be seen from the table that pearl millet surpassed sudan grass for most of the forage quality parameters studied. Both the application of chemical fertilizers and seed treatment with specific bacteria before sowing increased the digestibility percentage.

Table 3

Fresh and dry forage yield (t/ha) of sudan grass and millet as affected by fertilization treatments (combined analysis of two years)

Crop	Fertilization treatments	Fresh forage yield			Dry forage yield		
		1st cut	2nd cut	Total	1st cut	2nd cut	Total
Sudan grass	Organic manure	33.17	35.29	68.48	24.89	26.47	51.36
	Chemical fertilizers	34.81	37.31	72.12	26.11	27.98	54.09
	Organic + biofertilizers	36.62	39.24	75.86	27.47	29.43	56.90
	Chemical + biofertilizers	36.85	38.62	75.47	27.64	28.97	56.61
	Organic + biofertilizers + chemical fertilizers	36.95	40.12	77.07	27.71	30.09	57.80
	General mean	35.68	38.12	73.79	26.76	28.59	55.35
Pearl millet	Organic manure	33.95	35.83	69.78	24.46	25.87	50.33
	Chemical fertilizers	35.24	38.07	73.31	25.43	27.55	52.98
	Organic + biofertilizers	44.45	44.90	89.35	32.34	32.68	65.02
	Chemical + biofertilizers	37.86	42.29	80.15	27.40	30.72	58.12
	Organic + biofertilizers + chemical fertilizers	44.40	44.76	89.16	32.30	32.57	64.87
	General mean	39.18	41.17	80.35	28.39	29.88	58.26
LSD$_{5\%}$	Crop	2.14	1.16	4.22	1.33	1.12	2.17
	Fertilization treatments	1.33	1.78	1.16	1.46	1.36	1.28
	Interaction	2.35	1.88	2.22	1.14	2.14	2.22

Table 4

Mean values (%) of forage quality parameters in the 2nd cut of sudan grass and pearl millet grown in calcareous soil, as affected by fertilization treatments (combined analysis of two years)

Crop	Fertilization treatments	Crude protein	Fibre	Ash	Fat	Sol. carb.h.	Digestibility	
							1st cut	2nd cut
Sudan grass	Organic manure	7.2	30.7	10.8	1.7	49.60	56.7	48.7
	Chemical fertilizers	6.0	30.4	10.2	1.2	52.20	54.2	47.3
	Organic + biofertilizers	7.8	30.9	10.8	1.9	51.40	57.8	49.6
	Chemical + biofertilizers	6.8	31.6	10.8	1.9	48.90	57.9	49.8
	Organic + bio. + chem. fertilizers	8.0	31.7	10.9	1.9	47.50	57.8	49.9
	General mean	7.2	31.1	10.7	1.7	49.92	56.9	49.1
Pearl millet	Organic manure	8.2	30.4	9.8	1.8	49.80	60.1	57.6
	Chemical fertilizers	8.7	29.8	9.2	1.9	50.40	60.5	58.9
	Organic + biofertilizers	8.4	29.9	9.4	1.9	49.60	62.7	58.9
	Chemical + biofertilizers	8.6	29.9	9.2	1.9	50.65	66.8	60.8
	Organic + bio. + chem. fertilizers	9.3	29.1	9.5	1.8	50.30	63.4	57.6
	General mean	8.6	29.8	9.4	1.9	50.15	62.7	58.8

Sol. carb.h.: soluble carbohydrates

Conclusions

Sudan grass and millet will continue to be important food grain crops grown under a wide range of conditions. Their wide adaptation, due to their genetic composition, their tolerance to numerous biotic stress factors, and their ability to produce high forage yields with good quality, has the potential to make them important forage crops for diverse agro-ecological conditions. In the present trials, the maximum green forage yield was 89.16 and 77.07 t/ha for millet and sudan grass, respectively. In view of global warming, increasing water shortages and deteriorating natural resources there is a growing demand for alternative forage resources of high quality to meet the forage requirements of the increasing livestock population. Sudan grass and millet can be recommended as useful crop options, and moderate agriculture practices could further improve their yield and quality.

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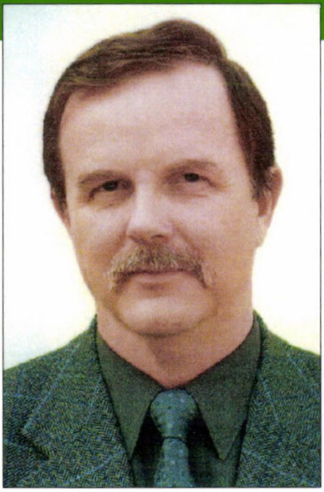

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BIOTECHNOLOGY AS A USEFUL TOOL FOR NUTRITIONAL IMPROVEMENT OF CEREAL-BASED MATERIALS ENRICHED WITH POLYUNSATURATED FATTY ACIDS AND PIGMENTS

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The fungi *Thamnidium elegans,* as a producer of γ-linolenic acid (GLA), and *Mortierella alpina,* as a producer of dihomo-γ-linolenic acid (DGLA), arachidonic acid (AA) and eicosapentaenoic acid (EPA), and the yeasts *Rhodotorula glutinis* and *Sporobolomyces roseus*, as producers of β-carotene, torulene and torularhodin, were tested for their ability to utilize cereal substrates during solid state fermentations (SSF). Depending on the strain and conditions, the cereal materials were effectively enriched with polyunsaturated fatty acids (PUFAs) or carotenoids. These naturally prepared bioproducts could find applications in food, feed, biomedical, pharmaceutical and veterinary fields.

Key words: cereals, solid state fermentation, polyunsaturated fatty acids, carotenoids

Introduction

Cereals represent a major food supply for humanity. Although these sources are rich in proteins and carbohydrates, many of them are deficient in several essential nutrients, such as polyunsaturated fatty acids (PUFAs) and carotenoid pigments. PUFAs, which have unique structural characteristics regulating the architecture, dynamics and permeability of membranes, are precursors of a wide variety of metabolites (such as prostaglandins, leukotrienes and hydroxy-fatty acids). PUFA deficiencies lead to abnormalities in the skin, nervous system, immune and inflammatory systems, cardiovascular system, endocrine system, kidneys, respiratory and reproductive systems (Gill and Valivety, 1997). There is also an increased interest in carotenoids as natural antioxidants and free radical scavengers for their ability to reduce and alleviate chronic diseases, various pathological stages and aging (Fraser and Bramley, 2004).

One way of enhancing the content of PUFAs and/or pigments in a cereal diet is to add these compounds to food or feed. Unfortunately, the inadequacy of natural sources rich in PUFAs and pigments does not allow them to be used in large quantities as food/feed supplements. In addition, PUFAs or carotenoids prepared synthetically have been strictly regulated in recent years. Another approach may be the application of gene engineering techniques in order to prepare new cereal varieties with the desired fatty acid or pigment profile. However, these methods are limited due to the difficulty of gene transformation in various cereals and the fact that transgene cereals containing PUFA or carotenoids are not approved for application in the food industry in many countries.

One attractive possibility for enhancing the content of PUFAs or carotenoids in cereals is based on the biotechnological transformation of cereal materials by solid state fermentations (SSF). SSF is characterized as a process in which microorganisms grow on a moist solid substrate in the absence of free water, simulating the fermentation reactions occurring in nature (Pandey, 2003). A Slovak team has pioneered the SSF process, in which microorganisms belonging to the *Mucoraceae* easily and efficiently utilized cereals containing starch, proteins and low lipid amounts and accumulated lipids with dietetically valuable PUFAs (Slugeň et al., 1994). Because these fungi simultaneously decrease the anti-nutrient compounds in the substrates (e.g. phytic acid) and partially hydrolyse substrate biopolymers, the pre-fermented mass with a high content of PUFAs may be used as an inexpensive food and feed supplement. Thus, SSF is a powerful tool for the effective utilization of agro-materials (e.g. cereals) and their conversion to various types of value-added bioproducts with the desired properties and functions (Čertík et al., 2002; 2006; Rapta et al., 2005).

This paper deals with the effectivity of several lower filamentous fungi or yeasts to synthesize various PUFAs or carotenoids, respectively, during their growth on cereals. Such naturally prepared cereal-based bioproducts enriched with either PUFAs or carotenoid pigments may be used as an inexpensive food and feed supplement. Thus, the association of selected microorganisms with solid state fermentations has created new prospects for the economic competitiveness and market of cereal-based bioproducts containing PUFAs or carotenoids.

Materials and methods

Microorganisms

Thamnidium elegans CCF 1456 and *Mortierella alpina* CCF 185 were obtained from the Culture Collection of Fungi (Charles University, Prague, Czech Republic). The cultures were maintained on modified Czapek-Dox agar slants with yeast extract (2.5 g/l) at 4°C. Yeast strains *Rhodotorula glutinis* CCY 20-2-26 and *Sporobolomyces roseus* CCY 19-6-4 were obtained from the Culture Collection of Yeasts (CCY; Institute of Chemistry, Slovak Academy of Sciences, Bratislava) and maintained on malt slant agar at 4°C.

Substrates and cultivation conditions

Depending on the microorganism, various types of substrates were employed during the SSF experiments. Spent malt grains (SMG) were added to some substrates and served as an internal support. Autoclavable microporous polypropylene bags (160 × 270 mm^2) were filled with 10 g of dry substrate [wheat sprout/SMG (3:1, w/w), wheat bran/SMG (3:1, w/w), rye bran/SMG (3:1, w/w), rice, peeled barley, sesame seeds, peeled barley/linseed oil/spent malt grains (0.5:1:3, w/w), wheat flour/linseed oil/yeast extract/spent malt grains (0.5:2:0.1:3, w/w)], moistened by the addition of 10 ml distilled water, soaked for 2 h at laboratory temperature and sterilized in an autoclave (120 kPa, 120°C, 20 min). Bags filled with oat flakes or noodles were first sterilized and then moistened with 10 ml sterile distilled water. The substrates were inoculated and mixed with 2 ml of either fungal or yeast spore suspension ($1–2.10^6$ spores per mL). Inoculated substrate was spread in the bags to obtain a substrate layer of about 1 cm and incubated statically at 24°C for 4–6 days (*T. elegans, R. glutinis, S. roseus*) or 10–14 days (*M. alpina*). Triplicate SSF experiments were prepared for each substrate to assess reproducibility, and average results are presented.

Lipid extraction and fatty acid analysis

Pre-fermented cereal materials (bioproducts) were gently dried at 65°C for 10 h and weighed. Lipids from homogenized bioproducts were isolated with chloroform/methanol (2:1, v/v) and purified according to Čertík et al. (1996) and total lipids were determined gravimetrically. The fatty acids of total lipids were analysed as their methyl esters (Christopherson and Glass, 1969) by gas chromatography according to Čertík et al. (2006). The gas chromatograph (GC-6890 N, Agilent Technologies) was equipped with a capillary column DB-23 (60 m × 0.25 mm, film thickness 0.25 μm, Agilent Technologies) and a FID detector (constant flow, hydrogen 35 ml/min, air 350 ml/min, 250°C). Analyses were carried out under a temperature gradient (130°C for 1 min; 130–170°C at program rate 6.5°C/min; 170–215°C at program rate 2.7°C/min; 215°C for 7 min; 220–240°C at program rate 2°C/min; 240°C for 2 min) with hydrogen as a carrier gas (flow 2.1 ml/min, velocity 49 cm/s, pressure 174 kPa) and a split ratio of 1/50 (inlets: heater 230°C, total hydrogen flow 114 ml/min, pressure 174 kPa). The fatty acid methyl ester peaks were identified by authentic standards for a C_4–C_{24} fatty acid methyl ester mixture (Supelco, USA) and quantified by an internal standard of heptadecanoic acid (C17:0, Supelco, USA). The fatty acid concentration was evaluated with ChemStation software B0103 (Agilent Technologies, USA).

Pigment isolation and analysis

Pigments from homogenized bioproducts were isolated with dimethylsulphoxide (DMSO) and analysed by high-performance liquid chromatography (HPLC). Analysis was carried out with an HP 1100 chromatograph (Agilent) equipped with a UV-VIS detector. Pigment extracts in DMSO (10 μl) were injected onto a LiChrospher® 100 RP-18 (5 μm) column (Merck). The solvent system (flow rate 1 ml/min) consisted of solvents A, acetonitrile/water/formic acid 86:10:4 (v/v/v), and B, ethyl acetate/formic acid 96:4 (v/v), with a gradient of 100% A at 0 min, 100% B at 20 min, and 100% A at 30 min.

Results and discussion

Biotechnological processes must be highly effective and competitive with other commonly used techniques in order to attain commercial feasibility (Čertík, 2008). Emphasis is put on SSF strategies that can lead to bioproducts with high PUFA and/or pigment contents. Because SSF can be carried out on a variety of agricultural materials and residues that have limited nutritional value, it is necessary to optimize the potential of microorganisms for transformation of these substrates into the desired metabolites.

Two lower filamentous fungi, *Thamnidium elegans* and *Mortierella alpina*, with the appropriate capacity to synthesize various PUFAs, were used for the conversion of numerous agroindustrial substrates to bioproducts enriched with gamma-linolenic acid (GLA), dihomo-gamma-linolenic acid (DGLA), arachidonic acid (AA) and eicosapentaenoic acid (EPA), while the yeast species *Rhodotorula* and *Sporobolomyces* were employed for the formation of carotenoids, such as β-carotene, torulene and torularhodin.

Bioproducts enriched with GLA

T. elegans effectively utilized various types of cereals and other materials and enriched them with oil containing GLA. This strain usually covered the substrates 2 days after inoculation and the whole cultivation took 4–6 days to complete. Spent malt grains served as an internal support and their ratio to the substrates was optimized previously (Čertík et al., 2006). The content of GLA in the bioproducts after the pre-fermentation of the materials is shown in Table 1. A mixture of wheat bran/spent malt grains (3:1, w/w) was found to be the best substrate and its solid state fermentation by *T. elegans* resulted in a bioproduct containing 0.5% GLA. Further improvement in GLA formation was achieved by the physiological regulation of the SSF process employing the following steps: a) gradual elevation of the carbon/nitrogen ratio with the addition of glucose or whey (Slávikova et al., 2002a); b) optimization of water activity, temperature and oxygen availability (Slavikova et al., 2002b); c) transformation of exogenously added oils consisting of linoleic acid as a precursor of GLA (Sláviková and Čertík, 2005). The final pre-fermented bioproduct contained 25% lipid. The total GLA amount yielded was almost 10 g/kg bioproduct. *T. elegans* was also used as a production microorganism by other authors and its growth on pearl barley or apple pomace with the addition of peanut oil resulted in 8.0 or 8.7 g GLA/kg bioproduct, respectively (Conti et al., 2001).

Bioproducts enriched with AA

Solid-state fermentations were also employed to improve the market for fungal AA-rich bioproducts. Screening of many fungi has resulted in the selection of *M. alpina*, which has also been used for the preparation of microbial oil with high AA content by submerged fermentations (Čertík and Shimizu, 1999). The growth of this fungus on agroindustrial materials during SSF was much slower compared with *T. elegans,* and pre-fermentation was completed after 10–14 days. Nevertheless, the search for an optimal substrate revealed that *M. alpina* satisfactorily converted a mixture of wheat bran/spent malt grains (3:1, w/w), leading to a bioproduct with 4.2% AA (Table 1).

Table 1

Lipid content, γ-linolenic acid (GLA) and arachidonic acid (AA) formation by solid state fermentation of *Thamnidium elegans* and *Mortierella alpina* grown on various cereal substrates. The substrates contain neither GLA nor AA

Substrate	*Thamnidium elegans*			*Mortierella alpina*		
	Lipid	GLA		Lipid	AA	
	(% in BP)	(% in TL)	(g/kg BP)	(% in BP)	(% in TL)	(g/kg BP)
Rice	4.1	4.2	1.2	8.1	31.1	21.4
Wheat sprout/SMG^a (3:1)	6.8	8.6	4.5	10.3	42.7	36.1
Rye bran/SMG^a (3:1)	5.4	9.5	3.8	9.2	34.4	21.9
Wheat bran/SMG^a (3:1)	4.7	14.3	5.0	11.2	45.3	42.3
Peeled barley	2.4	7.8	1.3	5.4	37.6	16.2
Oat flakes	9.3	6.4	4.7	10.7	35.7	31.2

SMG: spent malt grains; BP: bioproduct; TL: total lipid

Bioproducts enriched with DGLA

Basic cultivation of *M. alpina* usually leads to a standard fatty acid profile with a predominant concentration of AA and only a low level of DGLA. Since the bioconversion of DGLA to AA is catalysed by Δ^5 desaturase, the inhibition of this metabolic step is accompanied by a rapid increase in the DGLA/AA ratio. This strategy, which was successfully employed for DGLA overproduction in submerged cultivations (Shimizu et al., 1993), was also applied during SSF for the preparation of bioproducts enriched with DGLA by *M. alpina*. Figure 1a indicates that the addition of sesame seeds (containing sesamin analogues, known to be effective inhibitors of Δ^5 desaturase) to peeled barley rapidly enhanced both the DGLA level in the bioproduct and the DGLA/AA ratio. However, when *M. alpina* utilized crushed sesame seeds alone (characterized by a sufficient amount of saccharides, proteins and lipids), the DGLA/AA ratio in the bioproduct after 13 days of cultivation reached a value of 2.9, corresponding to almost 20 g DGLA/kg bioproduct (Fig. 1a). Subsequent cultivation for a further 5 days led to a final 21.3 g DGLA/kg bioproduct (Fig. 1a).

Bioproducts enriched with EPA

An SSF process was developed to prepare EPA-rich bioproducts using fungi that can rapidly utilize, incorporate and modify exogenously added oils. Linseed oil consists of about 57% α-linolenic acid, which is a precursor of PUFAs belonging to the ω-3 fatty acid family. Because *M. alpina* possesses an appropriate enzymatic apparatus for the possible transformation of α-linolenic acid to EPA (Čertík et al., 1998), linseed oil was added to the substrate with the aim of shifting the formation of ω-6 fatty acids (AA) to ω-3 fatty acids (EPA). This effort led to the pre-fermentation of an optimized mixture of peeled barley/linseed oil/spent malt grains (0.5:1:3, w/w) by *M. alpina*, which simultaneously yielded 23.4 g EPA/kg bioproduct (17.8% EPA in oil) and 36.3 g AA/kg bioproduct (27.6% AA in oil) (Fig. 1b). Thus, this strategy allows the preparation of oils with a desirable ω-6/ω-3 PUFA ratio, finally leading to more beneficial human applications.

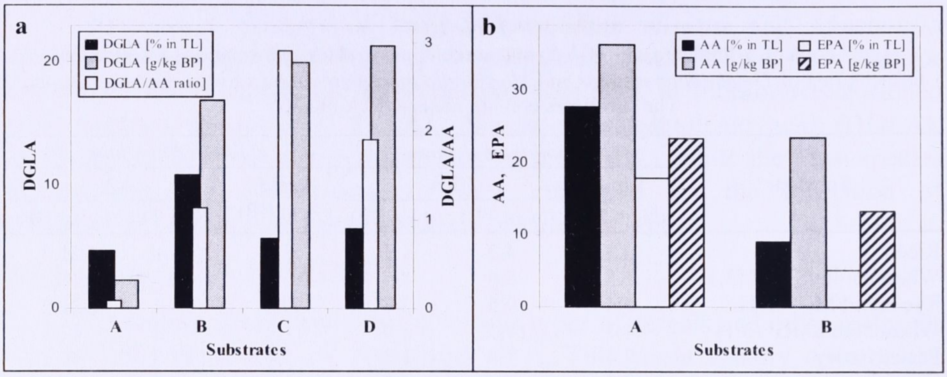

Fig. 1. Dihomo-γ-linolenic acid (DGLA), arachidonic acid (AA) and eicosapentaenoic acid (EPA) content in total lipids (TL) and bioproduct (BP) after solid state fermentation of *Mortierella alpina* grown on various substrates (substrates contain neither DGLA, AA nor EPA). 1a: A – peeled barley, B – peeled barley/sesame seeds (1:1, w/w), C – crushed sesame seeds (13 days SSF), D – crushed sesame seeds (18 days SSF). 1b: A – peeled barley/linseed oil/spent malt grains (0.5:1:3, w/w), B – wheat flour/linseed oil/yeast extract/spent malt grains (0.5:2:0.1:3, w/w)

Bioproducts enriched with carotenoid pigments

A number of pigment-forming yeast species and cereal materials have been screened for carotenoid accumulation during SSF. Yeast strains of the genera *Rhodotorula* and *Sporobolomyces* were selected as producers of β-carotene, torulene and torularhodin. Table 2 shows that both yeast strains enriched the cereal substrates mainly with β-carotene and torularhodin. It should be noted that during the growth of these yeasts in liquid media, β-carotene and torulene were synthesized as the principal pigments. The rapidly improved formation of torularhodin by yeasts (up to 55 and 74% of total carotenoids, respectively) during the utilization of wheat bran/SMG (3:1) and oat flakes might be useful for the overproduction of this pigment. In addition, the physiological regulation of the submerged fermentation process by various stress conditions (heavy metals, hydrogen peroxide, salt, light and solvents) resulted in a significant increase in the pigment yield (Márová et al., 2004; Breierová et al., 2005). Such treatment, with the aim of activating the biosynthesis of carotenoids by yeasts grown on cereals during SSF, is the subject of further studies.

Table 2

Carotenoids profile in bioproducts after solid state fermentation of *Rhodotorula glutinis* and *Sporobolomyces roseus* grown on various cereal substrates. The substrates do not contain carotenoids

Strain	Substrate	Carotenoids in bioproduct (% of total carotenoids)		
		β-carotene	Torulene	Torularhodin
Rhodotorula glutinis	wheat bran/SMG (3:1)	42.3	2.8	54.9
	oat flakes	47.5	0.8	51.7
Sporobolomyces roseus	wheat bran/SMG (3:1)	26.2	0.7	73.1
	oat flakes	36.6	0.1	63.3

SMG: spent malt grains

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MARKER-ASSISTED SELECTION FOR THE DEVELOPMENT OF IMPROVED BARLEY AND WHEAT LINES

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Marker-assisted selection (MAS) is an efficient modern method for transferring alleles or specific chromosome segments including important agronomic traits into elite cultivars. This approach makes genotypic selection possible, whereby the selection process is more effective. The Research Institute of Plant Production Piešťany uses genetic markers linked to important traits in the following pre-breeding programmes: 1. development of winter barley lines resistant to BaYMV/BaMMV, 2. development of spring barley lines resistant to BYDV, 3. development of winter wheat lines resistant to leaf rust (gene pyramiding), 4. improvement of wheat quality by new combination(s) of known HMW-GS and/or by introduction of novel HMW-GS alleles. Several hundreds of genotypes are usually analysed for the presence or absence of linked molecular markers and selected for use in breeding programmes.

Key words: marker-assisted selection (MAS), molecular breeding, gene pyramiding, *rym* genes, *Ryd2* gene, *Lr* genes, *Glu* alleles

Introduction

Traditionally, plant breeders have selected plants based upon their visible or measurable characteristics. One of the most powerful tools of biotechnology for plant breeding is marker-assisted selection (MAS). Molecular markers closely linked to genes of interest enable the early, reliable and rapid evaluation of individuals possessing the desired alleles during the breeding process, making it faster and more efficient. These markers are particularly useful for the incorporation of disease resistance genes that cannot be easily screened and for accumulating them within the same genotype to achieve durable resistance through the process of gene pyramiding (Ordon et al., 1999). An additional advantage of MAS incorporation into breeding programmes is that very different types of traits, e.g. disease resistance, quality, and others can be detected. The focus in the present work is on the molecular breeding of barley and wheat to create lines resistant against pathogen-based diseases and with improved quality traits.

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The soil-borne barley yellow mosaic virus complex (BaMMV, BaYMV-1, BaYMV-2) causes serious yield losses in winter barley production in East Asia and Europe. These viruses are naturally transmitted by the soil-borne fungus *Polymyxa graminis* Led. Chemical treatments against them or against disease symptoms are neither efficient nor acceptable for ecological and economic reasons (Ordon et al., 1999). The most common approach for the prevention of infection by these viruses is the introgression of resistance genes into breeding lines and cultivars (Okada et al., 2003). An attempt was thus made to create resistant winter barley lines by introducing the resistance genes *rym4*, conferring resistance against BaYMV and BaMMV (Friedt et al., 1990), and *rym11*, conferring resistance against BaYMV-2 (Bauer et al., 1997).

Barley yellow dwarf virus (BYDV) has a wide host range including wheat, barley, oats, and many cultivated and wild grasses. The symptoms of BYDV are highly variable even within a cereal crop. Plant dwarfing is a common feature of the disease in all crops. The virus is transmitted by infected aphid vectors. Resistance against BYDV in barley is provided by the gene *Ryd2*, identified for the first time in 1959 by Rasmusson and Schaller in Ethiopian spring barley lines (Collins et al., 1996; Jefferies et al., 2003). The majority of spring barley cultivars grown in Central Europe are susceptible or very susceptible to infection with the PAV strain prevalent in this region (Ovesná et al., 2000). The introduction of the *Ryd2* gene is generally regarded as highly beneficial, but the effects of this gene in different genetic backgrounds may be different. Work is currently underway on the transfer of this effective resistance gene into Slovakian spring barley cultivars.

The yield of wheat may be reduced by different fungal diseases each year. Among them, leaf rust is the most frequent and widespread in Slovak cereal growing areas. Leaf rust on wheat (*Triticum aestivum* L.), caused by *Puccinia recondita* f. sp. *tritici*, is adapted to a wide range of climates. Resistance against this fungus is based on effective leaf rust (*Lr*) resistance genes. There are currently more than 50 different known *Lr* genes, but the most effective are *Lr9, Lr19, Lr24, Lr28* and *Lr35*. The results of virulence analysis for 16 resistance genes in the Czech Republic during the years 2001–2004 confirmed the high effectiveness of the *Lr19* gene and the slightly lower efficiency of *Lr24* (Hanzalová and Bartoš, 2006). The present aim is to transfer the effective resistance genes *Lr19*, *Lr24* and *Lr35* into wheat genotypes with no or limited resistance to leaf rust, but well adapted to Slovak soil and climatic conditions.

Another important topic in wheat is the quality of the grain. The glutenins are one of the major components of storage proteins in wheat grain. These large, polymeric proteins have a very important role in determining dough characteristics (Payne, 1987; Manley et al., 1992; Weegels et al., 1996). Genes coding for high molecular weight glutenin subunits (HMW-GS) are located at the *Glu-A1, Glu-B1* and *Glu-D1* loci of chromosomes 1AL, 1BL and 1DL, respectively, and are important for improving the bread-making quality of wheat. Six HMW-GS genes are present in hexaploid wheat, but only those coding for subunits 1Bx, 1Dx and 1Dy are constantly expressed. The 1By and 1Ax subunits are present in some cultivars, while 1Ay subunits are not expressed in bread wheat. Nevertheless, the y-type subunit at the *Glu-A1* locus was reported in one Swedish wheat cultivar (Johansson and Svensson, 1995). Efforts are underway to use protein markers to develop near-isogenic wheat lines with higher sedimentation value, greater dough strength and better bread-making quality. It is hoped to develop lines with a new combination of HMW-GS (21*, 7+8, 5+10) that has not previously been present in Slovak wheat cultivars. It is also planned to create other wheat lines with novel, unknown HMW-GS originating from the landraces Eritrospermum 917 and Kotte.

Materials and methods

Barley and wheat populations were created by classical hybridization and MAS was used for indirect selection. Elite Slovak and Czech cultivars and lines were used as acceptors of interesting genes. Newly created lines carrying markers linked to desired genes were then tested for resistance and for agronomic and technological traits.

Genomic DNA was isolated from segments of young leaves using Plant DNAzol (Invitrogen). PCR amplifications using specific primers were carried out in a PTC-200 thermal cycler (MJ-Research) and the amplification products were separated in agarose gels.

The cultivar Romanze was used as the donor of the *rym4* gene in breeding winter barley for resistance to BaYMV/BaMMV. The genotypes Copia, Kamil and Luxor, and the breeding line KM-104 were used as acceptors of this gene. The presence or absence of the *rym4* gene in the F_2 progeny was determined with the codominant STS marker derived from the RFLP marker MWG838 linked to this gene (Tuvesson et al., 1998). The lines were also tested in naturally infested field trials. Re-tested lines were then crossed with the landrace Russia 57, used as the donor of the *rym11* gene. The progenies were evaluated with the codominant SSR marker HVM3, linked to the *rym11* gene (Bauer et al., 1997), and with the STS marker, linked to the *rym4* gene.

Spring barley populations were based on cultivars Nitran and Ludan and line SK 5104 as acceptors, and Sutter and Shannon as donors of the *Ryd2* gene, which confers effective resistance against BYDV. Indirect selection was made using the dominant ASPCR Ylp marker for the *Ryd2* associated allele of the *Ylp* gene, which is closely linked to the *Ryd2* gene (Ford et al., 1998).

Effective resistance genes against leaf rust, *Lr19*, *Lr24* and *Lr35*, were transferred into the elite winter wheat cultivars Hana, Astella, Klea, Brea and Alka. The cultivar Agrus was used as a donor of the *Lr19* gene, and near-isogenic lines based on the cultivar Thatcher as donors of the *Lr24* and *Lr35* genes. The presence of the *Lr19* and *Lr 24* genes was detected using dominant STS markers according to Prins et al. (2001) and Schachermayr et al. (1995). A dominant SCAR marker was used to detect the *Lr35* gene, as reported by Gold et al. (1999).

Glutenins were extracted, electrophoresed and visualized according to the standard SDS-PAGE technique for wheat (Wrigley, 1992). Glutenin patterns were evaluated with a densitometer (ImageMaster DTS, Pharmacia Biotech). Homogeneity or heterogeneity, respectively, in the protein composition was studied by comparison of the complete protein patterns revealed by SDS-PAGE. High molecular weight glutenin subunits (HMW-GS) were identified according to Payne and Lawrence (1983). The Glu-quality score was calculated according to Payne (1987). The genotype Kotte was used as donor for new alleles encoding HMW-GS at the locus *Glu-1B* and a Swedish bread wheat line was used as donor for the *21** allele at *Glu-1A*. Cultivars Hana, Danubia, Elpa, Torysa, Simona and Klea were used as recurrent parents.

Results and discussion

Development of winter barley lines resistant to BaYMV/BaMMV

The presence of resistance gene *rym4* was confirmed by both molecular and phytopathological testing. Segregation in the F_2 progeny suggested that resistance was based on a single gene (Ordon and Friedt, 1993; Konishi et al., 1997; Pellio et al., 2000). Winter barley lines carrying the molecular marker linked to resistance gene *rym4* were tested under field conditions for the main agronomic characteristics. Lines from the combinations (KM 104 × Romanze) F_3 and (Kamil × Romanze) F_3 gave favourable values of thousand-kernel weight and kernel weight per plant. Some of these lines were crossed with the landrace Russia 57 as the *rym11* donor to accumulate two different genes into one line (Fig. 1). Plants possessing two markers for resistance genes *rym4* + *rym11* were selected from all four crosses. Six plants (4.3%) possessing both genes were identified in lines of (Copia × Romanze) × Russia 57, nine (6%) in lines of (KM 104 × Romanze) × Russia 57 and (Luxor × Romanze) × Russia 57, and three (2%) in lines of (Kamil × Romanze) × Russia 57, among the 150 plants analysed for all the crosses. The gene *rym4* is located on the long arm of chromosome 3 and gene *rym4* on the long arm of chromosome 4, so it should be possible for them to combine. A low recombination rate is expected between *rym9* and *rym11*, as they are both located on chromosome 4HL (Ordon et al., 1999). Based on PCR markers, different strategies are applied for combining different genes (pyramiding). Pellio et al. (2000) used DH lines and the marker-based identification of DHs homozygous for *rym4, rym9* and *rym11*. F_1 plants [e.g. (*rym4* × *rym9)* × *(rym4* × *rym11)*] were inter-crossed and about 100 kernels were tested with markers to identify those that were homozygous at one resistance locus and heterozygous at the other (6.25%). During the incorporation of the resistance gene, especially from landrace Russia 57, negative traits are also transmitted to the offspring (e.g. low yield, tall stem), which must be suppressed by the backcrossing procedure.

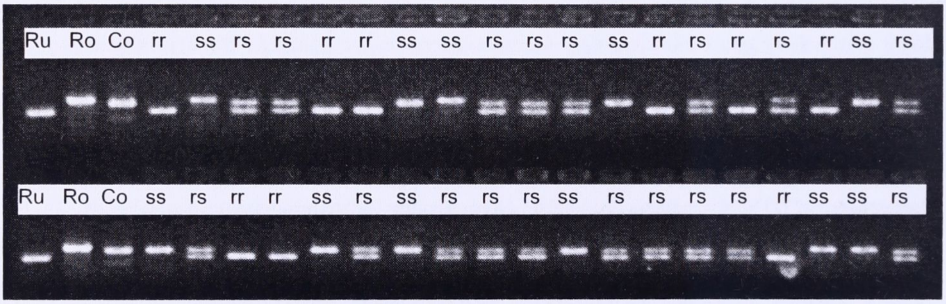

Fig. 1. Segregation of codominant DNA-SSR marker HVM3, linked to the *rym11* gene, in F_2 plants from the cross (Copia × Romanze) × Russia 57 (Ru = Russia 57, Ro = Romanze, Co = Copia, rr = resistant recessive homozygote, rs = susceptible heterozygote, ss = susceptible dominant homozygote)

Development of spring barley lines resistant to BYDV

The Ylp marker, which is efficient for distinguishing between the homozygous and heterozygous state, was used for the marker-assisted selection of F_2 progenies. Ninety plants with marker-based resistance were selected out of 220 individually evaluated progenies from the F_2 generation. These individuals were also tested for powdery mildew resistance (*Blumeria graminis* f. sp. *hordei*). Plants resistant to both fungi and virus were selected from the crosses Nitran × Sutter and Ludan × Shannon. Resistant plants were included in a backcrossing programme after phytopathological testing and positive molecular selection. The Ylp marker was previously used in breeding winter and spring barley with resistance to BYDV in the Czech Republic (Ovesná et al., 2000), where the Ylp marker-based results corresponded with those of field resistance testing. Resistance gene *Ryd2* is widely used in barley breeding programmes. It has been noted that the presence of the *Ryd2* gene negatively influences important agronomic traits such as yield, kernel weight, time of heading, etc. in cultivated barley, but this information was not confirmed (Jefferies et al., 2003). The effect of resistance gene introgression on the malting quality and agronomic traits of acceptor varieties is very important. Yield traits were evaluated in the BC_2F_2 generation in the present work and were compared with the recurrent parent. An increase in the tiller number was recorded in lines of (SK 5104 × Sutter) BC_2F_2 and (Ludan × Shannon) BC_2F_2, where the number of kernels per spike of the recurrent parent was retained. The stems of lines from the cross (Nitran × Sutter) BC_2F_2 were 10 cm shorter compared to variety Nitran.

Development of winter wheat lines resistant to leaf rust

The leaf rust resistance genes *Lr19, Lr24* and *Lr35* are effective resistance genes of various origin. Genes *Lr19* and *Lr24* have been introduced into hexaploid wheat from the wild species *Thinopyrum ponticum* (syn. *Agropyrum elongatum*, 2n=10x) and gene *Lr35* from *Aegilops speltoides*. Backcrosses and MAS were used for the transfer of these *Lr* genes into the elite winter wheat cultivars Alka, Astella, Brea, Hana and Klea, which possess HMW-GS alleles with a positive effect on bread-making quality. Wheat populations carrying the seedling resistance genes *Lr19* and *Lr24*, and the adult-plant resistance gene *Lr35* were obtained. These genes were identified in segregating populations by DNA markers. Near-isogenic lines with *Lr35*, *Lr19* and *Lr24* genes in elite wheat cultivar backgrounds are used in gene pyramiding to develop a single line with all three genes. To date, backcrossing has been accomplished to BC_5–BC_6 and will be continued through cycle six, after which the populations will be inbred by self-pollination, selected using DNA markers and HMW-GS alleles, and tested for the presence of specific leaf rust resistance genes in phytopathological tests. The pyramiding of resistance genes improves the disease resistance capacity of the genotypes. Lines with the gene combinations

Lr13 and *Lr34*, *Lr13* and *Lr37* and *Lr34* and *Lr37* had a higher level of resistance than lines with individual genes (Kloppers and Pretorius, 1997). The combination of two or more resistance genes is often difficult, but the availability of molecular markers tightly linked to the desired genes can help in the selection of individuals with introduced genes within segregating populations. The development of genotypes by pyramiding genes through marker-assisted selection has been reported by several authors in wheat and rice (Hittalmani et al., 2000; Liu et al., 2000; Singh et al., 2001). Šliková et al. (2004) used markers to pyramid leaf rust resistance genes *Lr19* and *Lr24*, and Gupta et al. (2005) for genes *Lr9* and *Lr24* in wheat.

Development of wheat lines with novel combinations of HMW subunits and unknown HMW subunits

Selection for desirable HMW-GS such as 5+10 (coded by *Glu-D1d*), 21* (coded by *Glu-A1*) and 7+8 (coded by *Glu-B1*) was performed in early generations of the breeding material (Fig. 2). The aim of the work was to use protein markers in segregating populations, to fix unique combinations of glutenin alleles, and to eliminate unfavourable glutenin alleles in backcross cycles.

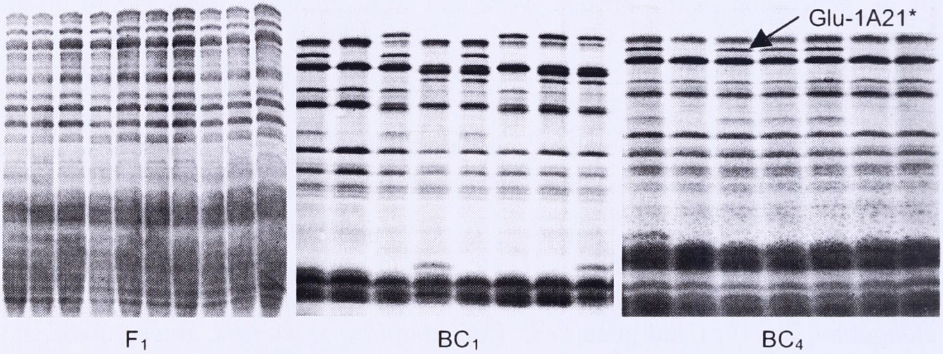

Fig. 2. Development of a near-isogenic line for HMW-GS 21*, 7+8 and 5+10 in the background of cultivar Hana using protein markers

The electrophoretic analysis of wheat glutenins in landraces allows novel HMW-GS alleles to be detected. A novel HMW-GS with electrophoretic mobility between HMW-GS 8 and 9, located at locus *Glu-1B*, was detected in one of the lines of landrace Eritrospermum 917 (Gregová et al., 1999). Another novel HMW-GS pair with electrophoretic mobility between HMW-GS 7 and 8, probably located at locus *Glu-1B*, was detected in one of the lines of landrace Kotte, and the relative molecular weights were calculated as 104 kDa and 120 kDa, respectively (Gregová et al., 2004). Other new HMW-GS alleles were detected at the loci *Glu-1B* (Gregová et al., 2006) and *Glu-1D* (in progress). The aim is to develop near-isogenic wheat lines with new HWM-GS pairs with electrophoretic mobility between HMW-GS 6 and 9 in Hana, Simona, Torysa, Elpa and Danubia backgrounds using protein markers. These near-isogenic lines will be valuable for the future assessment of the effects of the 21* and 6.2+8.3 subunits for agronomic performance and end-use quality.

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APPLICATION OF MOLECULAR MARKERS IN PARENTAL SELECTION IN SOYBEAN

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The rate of genetic gain in the quantity and quality of soybean grain depends considerably on the genetic diversity of the selected parental components. Genetic diversity assessment is a crucial aspect of breeding that maximizes genetic improvement. The objectives of this study were to evaluate the genetic diversity of the selected soybean germplasm using genetic markers, as well as to compare the effectiveness of breeding procedures with and without the use of genetic markers in parental selection. The genetic relationships within the selected soybean germplasm were estimated using 14 simple sequence repeats (SSRs). The agronomic performance (grain yield, protein and oil content in the grain) of the parental components and derived lines was determined in field trials. Based on SSR marker data and phenotypic data, an association was found between the agronomic performance of the derived lines and the genetic distance between the parental lines. Crosses between more diverse parents resulted in derived lines with greater values for grain yield and grain quality compared with the parents than crosses between similar parents. The results indicated the usefulness of genetic marker information in parental selection, contributing to breeding efficiency.

Key words: soybean, genetic diversity, parental selection, derived lines, SSRs, grain yield, grain quality

Introduction

Genetic improvement in soybean [*Glycine max* (L.) Merr.] based on conventional breeding strategies contributes to advances in production and in the food processing industry by developing high-yielding, high quality cultivars. The rate of genetic gain in the quantity and quality of soybean grain depends considerably on the genetic diversity of the selected parental components. Hence, the assessment of the genetic diversity of the available germplasm is a crucial aspect of soybean breeding for maximizing genetic improvement. Soybean breeders have traditionally used morphological and agronomic traits as well as pedigree information in genetic diversity evaluation. However, all these measures have limitations and they are not sufficient in themselves, particularly in closely related cultivars. Molecular markers have several advantages over the traditional phenotypic markers: accuracy, reliability, speed, indifference to the conditions under which the plants are grown and detectability in all stages of plant growth. Therefore, pedigree lineages, phenotypic and DNA markers together can provide more reliable information about the characterization, degree of diversity and genetic constitution of the existing germplasm, thus contributing to soybean breeding efficiency (Sneller, 1994; Doldi et al., 1997; Narvel et al., 2000; Chapman et al., 2003; Orf et al., 2004; Sneller et al., 2005; Rajcan et al., 2005).

In Croatia, the soybean breeding programme at the Agricultural Institute Osijek has made a fundamental and very significant contribution to the development, stability and improvement of soybean production. Continued genetic improvement has been accomplished through modern breeding strategies based on a combination of conventional breeding methods and modern laboratory analysis (Sudaric et al., 2006; 2007; Vrataric and Sudaric, 2000; Vrataric et al., 2004; 2005).

The present paper discusses the part of the soybean breeding programme at the Agricultural Institute Osijek related to the application of DNA markers to measure genetic diversity in parental selection. The objectives of the study were: *i*) to evaluate the genetic diversity within and between local and foreign soybean cultivars using polymorphism SSR markers; *ii*) to compare the effectiveness of breeding procedures with and without the use of genetic markers in parental selection.

Materials and methods

Molecular genetic analysis

The initial fingerprinting of 15 registered soybean cultivars developed at the Agricultural Institute Osijek, Croatia (OS-cultivars) was performed in collaboration with the molecular laboratory of the University of Guelph (Canada) using simple sequence repeats (SSR markers). Selected OS-cultivars differed for a wide range of agronomic and morphological traits. Besides OS-cultivars, fingerprinting was carried out for 51 soybean cultivars, mostly developed at the University of Guelph (CA-cultivars). All 66 cultivars tested were grown in a greenhouse under controlled conditions (air temperature: 20–25°C; air humidity: 80%) for DNA isolation. The equivalent of 30 leaf tissue samples was collected from each cultivar and lyophilized. DNA was isolated from the bulked lyophilized leaf tissue of the plants of each cultivar using the FastDNA kit from BIO 101 and amplified via a polymerase chain reaction (PCR) using 14 simple sequence repeats (SSR markers). Fourteen pairs of soybean primers flanking the microsatellite regions, coded as Satt 102, Satt 154, Satt 167, Satt 196, Satt 231, Satt 311, Satt 329, Satt 338, Satt 406, Satt 434, Satt 442, Satt 531, Satt 569 and Satt 598, were selected from the USDA-ARS Soybean Genome Database. PCR amplification was performed for each soybean genotype, using primers for each SSR locus. The DNA quality and concentration were evaluated by electrophoresis in 0.8% agarose gel stained with ethidium bromide (EtBr). The bands were considered and scored for each genotype and primer. The results were scored manually and converted to a present-absent scale (1 or 0 for each allele and genotype). A dendrogram was constructed using the unweighted pair-group mean arithmetic method (UPGMA) and drawn using TREE-VIEW software.

Field experiments

Within the framework of the scientific collaboration between the soybean breeding programmes of the Agricultural Institute Osijek and the University of Guelph a number of registered cultivars have been exchanged. The agronomic performance (grain yield, grain yield components, grain quality, plant lodging, maturity) of local (OS-cultivars) and foreign (CA-cultivars) soybean germplasm was determined in field trials conducted in Osijek (Croatia) during the period from 2002 to 2005. The first crosses between OS and CA cultivars, aimed to introgress foreign germplasm from CA-cultivars into OS-cultivars, were made in 2003 and designed only on the phenotypic level. After performing molecular analysis, parental combinations were designed from a combination of phenotypic and molecular marker data. The effectiveness of breeding procedures with and without the use of genetic markers in parental selection was evaluated in the F_4 generation, comparing mean values for grain yield and the protein and oil contents in the grain of parental components and derived lines.

Results and discussion

The UPGMA dendrogram for the 66 soybean cultivars (Fig. 1) demonstrated genetic relatedness between and within the OS and CA cultivars, distinguishing groups with maximum and minimum similarities. The genetic analysis did not show any correlation with similar morphology or geographical origin for the tested cultivars.

Target traits in the recombinants were earliness (typical of CA cultivars) and high grain yield or high grain quality. The agronomic values of the first lines (F_4 generation) derived from crosses carried out between OS and CA cultivars in 2003 are presented in Tables 1, 2 and 3. All the lines were earlier (desirable trait) in relation to the local (OS) parental line. A broad range of values were recorded for grain yield and grain quality. Thus, lines that were earlier than the local parental lines were found with desirable and undesirable values for grain yield (Table 1), grain protein content (Table 2) and grain oil content (Table 3). Comparing the parental components of these lines at the molecular level, an association was found between the agronomic traits of the derived lines and the genetic distance (genetic similarity) between their parental lines. Thus, in combinations where the parental components were less genetically distant (more genetically similar), the derived lines had lower values for the analysed traits than the local (OS) parent (undesirable recombination). However, if the parental components were more genetically distant (less genetically similar), the derived lines had higher values for grain quantity and quality than the local parental line (desirable recombination). From the practical point of view, this means that in combinations with more genetically distant parents, both the target traits were combined in the derived line and the breeding aim was achieved.

These results indicate the usefulness of genetic distance estimates between parents in predicting the genetic variance of agronomic traits. Some authors (Orf et al., 2004; Sneller et al., 2005) reported that crosses between more diverse elite parents resulted in a population with greater genetic variation for important agronomic traits than crosses between similar parents. Hence, the application of molecular markers and the use of the results of molecular genetic analysis in combination with phenotypic data on the genotypes could lead to the selection of parental combinations with the greatest potential to yield new, high-yielding, high quality cultivars. Therefore, genetic marker information in parental selection could have a favourable impact on breeding efficiency and contribute to the faster achievement of genetic improvement in soybean.

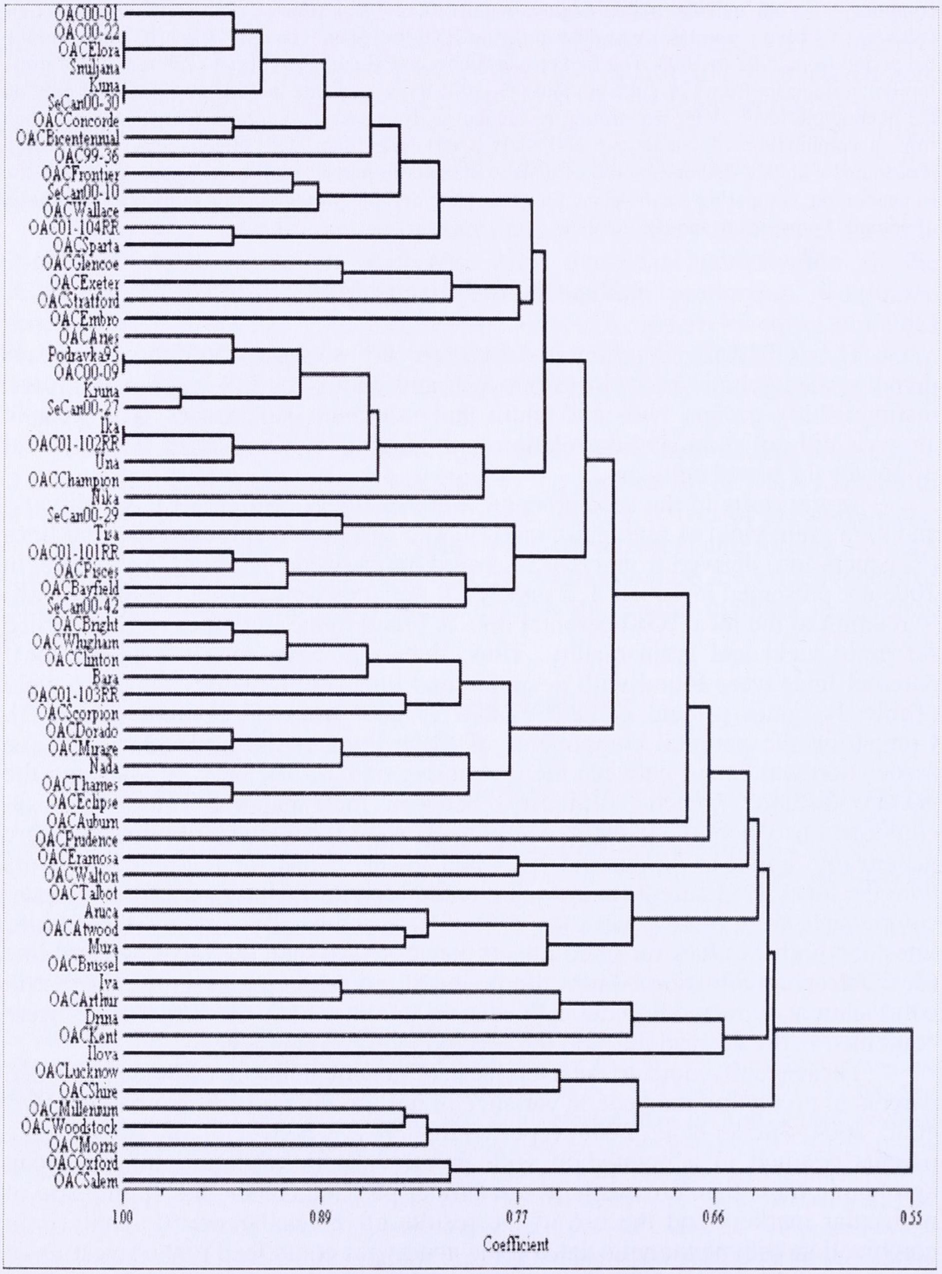

Fig. 1. Dendrogram of soybean genotypes based on the polymorphism of SSR markers

Table 1

Grain yield (t/ha) of parental and derived soybean lines (F_4 generation, Osijek, Croatia, 2007)

Line code	Female component	Male component	Derived line		
			Value	Deviation of	
				Female component	Male component
Small genetic distance between parental lines					
L-86-03	3.14 (CA)	3.62 (OS)	3.50	+ 0.36	– 0.12
L-104-03	3.14 (CA)	4.20 (OS)	3.65	+ 0.51*	– 0.55*
L-165-03	4.22 (OS)	3.67 (CA)	3.98	– 0.24	+ 0.31
L-224-03	4.22 (OS)	4.14 (CA)	4.20	– 0.02	+0.06
Large genetic distance between parental lines					
L-23-03	3.28 (CA)	3.66 (OS)	3.92	+ 0.64*	+ 0.26
L-96-03	3.28 (CA)	3.94 (OS)	4.20	+ 0.92*	+ 0.26
L-115-03	4.09 (OS)	3.89 (CA)	4.52	+ 0.43*	+ 0.63*
L-193-03	4.09 (OS)	3.67 (CA)	4.38	+ 0.29	+ 0.71*

$LSD_{0.05}$: 0.404

Table 2

Protein content in the grain (as a % of air-dry matter) of parental and derived soybean lines (F_4 generation, Croatia, Osijek, 2007)

Line code	Female component	Male component	Derived line		
			Value	Deviation of	
				Female component	Male component
Small genetic distance between parental lines					
L-48-03	38.51 (CA)	39.96 (OS)	39.40	+ 0.89*	– 0.56*
L-94-03	38.51 (CA)	40.50 (OS)	39.56	+ 1.05*	– 0.94*
L-136-03	40.88 (OS)	39.27 (CA)	40.35	– 0.53*	+ 1.08*
L-212-03	40.88 (OS)	40.52 (CA)	40.62	– 0.26	+ 0.10
Large genetic distance between parental lines					
L-11-03	38.87 (CA)	39.28 (OS)	39.43	+ 0.56*	+ 0.15
L-65-03	38.87 (CA)	39.61 (OS)	39.75	+ 0.88*	+ 0.14
L-170-03	40.06 (OS)	39.79 (CA)	40.18	+ 0.12	+ 0.39*
L-205-03	40.06 (OS)	40.16 (CA)	40.46	+ 0.40*	+ 0.30

$LSD_{0.05}$: 0.370

Table 3

Oil content in the grain (as a % of air-dry matter) of parental and derived soybean lines (F_4 generation, Osijek, Croatia, 2007)

Line code	Female component	Male component	Derived line		
			Value	Deviation of	
				Female component	Male component
Small genetic distance between parental lines					
L-26-03	22.15 (CA)	22.41 (OS)	22.30	+ 0.15	– 0.11
L-38-03	22.15 (CA)	22.28 (OS)	22.20	+ 0.05	– 0.08
L-111-03	22.20 (OS)	22.06 (CA)	22.16	– 0.04	+ 0.10
L-154-03	22.20 (OS)	22.00 (CA)	22.18	– 0.02	+ 0.18
Large genetic distance between parental lines					
L-30-03	22.54 (CA)	22.20 (OS)	22.48	– 0.06	+ 0.28
L-42-03	22.54 (CA)	22.41 (OS)	22.60	+ 0.06	+ 0.19
L-139-03	22.51 (OS)	22.36 (CA)	22.55	+ 0.04	+ 0.19
L-180-03	22.51 (OS)	22.24 (CA)	22.55	+ 0.04	+ 0.31

$LSD_{0.05}$: 0.348

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GRAPEVINE HABITUATION: UNDERSTANDING OF FACTORS THAT CONTRIBUTE TO NEOPLASTIC TRANSFORMATION AND SOMACLONAL VARIATION

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Two-dimensional gel electrophoresis coupled to protein microarray analysis was used to examine for the first time the molecular mechanisms of grapevine habituation (*Vitis vinifera* L., cv. Limberger) at both the proteome and the interactome level. The examination of 2-D maps derived from control and habituated cell cultures revealed the presence of 55 protein spots displaying a differential expression pattern. Using computational prediction methods, fundamental differences were found between eukaryotic interactomes. It was confirmed that all the predicted protein family interactomes (the full set of protein family interactions within a proteome) of six species are scale-free networks, and that they share a small core network comprising 16 protein families related to indispensable cellular functions predominantly involved in pathogenesis, apoptosis and plant tumorigenesis. There is molecular evidence suggesting that grapevine cells which have become habituated for one or more essential factors originated from heritable alterations in the pattern of gene expression and that they can, therefore, be used as a model for the study of cell differentiation and/or neoplastic transformation.

Key words: *Vitis vinifera*, proteomics, interactomics, bioinformatics, microarrays, expression profiling, electrophoresis, phytohormones

Abbreviations: BaP – benzylaminopurine, BSA – bovine serum albumin, NAA – naphthylacetic acid, PAGE – polyacrylamide gel electrophoresis, PhA – photosynthetically active, PhI – photosynthetically inactive

Introduction

A type of heritable cellular change, known as habituation (for review see Meins, 1982), occurs spontaneously in plant tissue and cell culture. It is the acquired ability of a population of cells to grow and divide independently of exogenously supplied growth regulators. Habituated tissues maintained in culture produce significant amounts of the growth factor to which they are habituated. Habituation results in the persistent biosynthesis of specific growth factors. The fact that intact plants produce auxin and cell-division factors suggests that habituation is due to the heritable expression of genes normally inactive in cultured cells. Habituation to auxin has been found to occur in a large number of plant species, including tobacco (Buiatti and Bennici, 1970), sunflower (Henderson, 1954), maize (Hawes et al., 1985), *Lilium longiflorum* (Sheridan, 1968) and grapevine (Morel, 1947). It has also been encountered for cytokinins, amino acids, and several vitamins (Kulescha and Gautheret, 1948; Gautheret, 1955; Ikeda et al. 1979). An imbalance of phytohormones in the medium, particularly auxins and cytokinins, is an important source of stress and has been linked to hyperhydricity, somaclonal variation, recalcitrance and habituation. All of these abnormalities are potentially very costly for the plant breeding industry. A comparison of the properties of habituated and crown gall cells leads to the conclusion that habituation represents a form of neoplastic transformation. Moreover, habituation as a tumorous and/or neoplastic transformation state is interchangeable with the normal state in plant cells.

To date, several habituated leaf (Hl) loci conferring habituation on tobacco (*Nicotiana tabacum*) leaf tissues have been identified (Hl-1, Hl-2 and Hl-3), two of which were reported to be meiotically transmissible (Meins and Foster, 1986; Meins, 1989). At present experimental evidence for the mechanism of habituation is scant. One approach towards the identification of the mechanism of habituation is a comparison of gene expression in habituated and non-habituated cell cultures. A transcriptome-based analysis of habituated and non-habituated *Arabidopsis thaliana* plant cell cultures revealed the differential expression of more than 800 genes, which included a 19-fold up-regulation of the transcript encoding the cytokinin receptor CRE1 (Pischke et al., 2006).

Since 1995, over 250 genomes have been completely sequenced (Shendure et al., 2004). The availability of such genomic sequence data allows us to conduct comparative genomics studies, yielding important information on developmental processes and disease defence mechanisms (Rubin et al., 2000; Eichler and Sankoff, 2003). Protein comparison using proteomes alone is, however, not fully sufficient to understand how the cellular machinery evolved over a long period of time. A step forward would be to look at all the interactions among them. An interactome is a whole set of molecular interactions in a cell. When used in terms of proteomics, it refers to the protein–protein interaction network (PPI) or the protein network (PN). The study of the interactome is called interactomics. Because the interactome considers the whole organism, there is a need to collect a massive amount of information. In the present case a computational prediction method was used, based on known protein structural interactions within and between complete genomes such as yeast, fly, worm, *Arabidopsis* and human, and their high throughput (HTP) maps, to analyse large-scale protein-protein interaction rules in grapevine. These studies may help to clarify the molecular mechanisms of neoplastic phenomena in plants and perhaps in animals.

Materials and methods

Plant material

Long-term cultivated callus cultures of grapevine (*Vitis vinifera* L., cv. Limberger) were used for all the experiments. These cultures were maintained under controlled environmental conditions at 25°C, 80% relative humidity, a photon flux density of 80 μmol m^{-1} s^{-1} and a 16/8 h photoperiod.

Habituation experiment

Sucrose, Gamborg vitamins, auxins and cytokinins were incorporated in Murashige & Skoog media at different concentrations and grapevine calluses were cultivated on these media for a period of 30 d. The experiments were then terminated, fresh weights were recorded and tissues were frozen for proteome analysis.

Sample preparation, protein expression profiling and comparative interactomics

The plant material was processed according to previously published procedures (Repka, 2006). Total protein content was estimated for each sample using BSA as the standard and the DC reagent (Bio-Rad, Richmond, USA). For analytical and/or preparative separations 2-D PAGE profiling was performed and the reproducibility of these profiles was confirmed by carrying out two independent experiments. After the completion of 2-D PAGE, these gels were fixed and silver stained according to the protocol of Rabilloud et al. (1992). Digitized images were obtained using the Kodak Images station 2000R at 60.5 μm resolution and analysed with the software tools included. Proteomics-based identifications using high-throughput microarrays, as well as the immunodetection of specific antigens, were conducted basically according to previously published protocols (Repka, 2006). To analyse protein–protein interaction networks, a threshold-free functional profiling analysis was performed within and among complete genomes, such as yeast, fly, worm, *Arabidopsis* and human, and their HTP maps. The following interactome databases were effectively examined to analyse the grapevine interactome: Database of interacting protein (DIP), GRID database, MIPS database, PSIMAP database (The first protein structural interactome database), InterPare database (A structural protein interfaceome database), Biomolecular Interaction Network Database (BIND), Online Predicted Human Interaction Database (OPHID), Human Protein Reference Database (HPRD/HPID), MINT, PINdb, IntAct (The molecular interaction database) and APID (Agile protein interaction DataAnalyzer), an interactive bioinformatics web-tool that integrates and unifies the main experimentally validated protein–protein interactions.

Results and discussion

High-resolution 2-D PAGE maps displaying up to 2,500 spots were visualized from control, auxin- and cytokinin-habituated samples. Figures 1A–C and 1D–F represent the 2-D protein patterns obtained from PhA and PhI calluses, respectively. Proteome maps of PhA and PhI habituated either for cytokinin or auxin revealed dramatically different expression patterns (Fig. 1B–C and E–F) when they were compared with the control (Fig. 1A, D). The number of up-regulated spots largely exceeded that of down-regulated ones. Fifty-five proteins showed an altered expression pattern due to the different metabolic state of both types of callus cultures. Figures 1B and E, representing callus cultures habituated for the BaP compound, exhibit a significantly changed pattern that is different from their control and/or auxin-habituated counterparts.

The same is true for the auxin-habituated cultures (Fig. 1C and F), where proteome changes differed from those in control and/or BaP-habituated ones. The BaP-habituated proteome map seems to be the most complex, indicating significant metabolic transformation of the tissue. These changes are in good accordance with a biochemical switch model for cell-heritable variation in the cytokinin requirement (Meins, 1989).

Two prominent spots in PhI calluses (Nos. 99 and 78; Fig. 1A–C, window a) exhibited no variation in the expression profile regardless of the habituation status. The differential accumulation of a number of small proteins was noted in PhI calluses (Fig. 1A–C, window b). Unlike the PhI callus cultures, other small groups of proteins were reproducibly expressed in both cytokinin- and auxin-habituated calluses (Fig. 1D and F, windows d and e, respectively). Interestingly, two other groups (Fig. 1D–F, windows f and c) displayed significant changes in their expression levels in habituated calluses compared with the control.

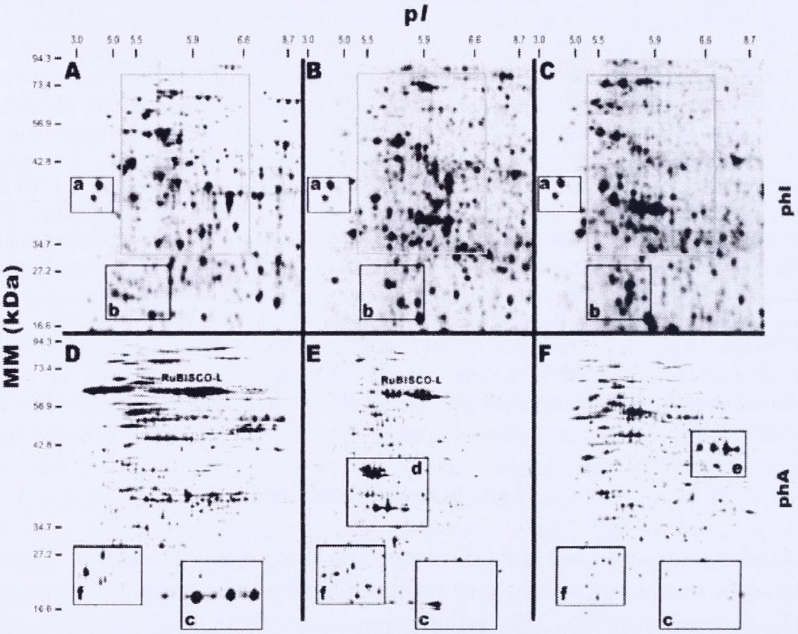

Fig. 1. Representative 2-D PAGE gels visualizing proteins from PhI and PhA calluses habituated either for cytokinin (B, E) or auxin (C, F) compared to control proteome maps (A, D). The first dimension was focused across a 3 to 10 pH unit range and the second electrophoretic dimension resolved proteins between 10 and 120 kDa. An equal amount (150 μg) of total protein extracts was loaded in each gel

Looking individually at the proteome maps from PhI calluses, nine protein spots (protein Nos. 94, 175, 264, 271, 323, 327, 331, 423 and 438) were induced from non-detectable levels and their expression patterns varied significantly between treatments (Fig. 2). Five other prominent protein spots (Nos. 659, 674, 681, 728 and 732) were also differentially expressed in PhA calluses derived from control and habituated cells (Fig. 3A). Immunoblot analysis of whole proteome maps with specific antibodies led to the identification of three 14-3-3 proteins in auxin-habituated calluses (spots Nos. 659, 674 and 681; Fig. 3B). Spot 659 was also identified immunospecifically in control calli. Further immunospecific analysis resulted in the identification of two small grapevine PRP-10 proteins (Nos. 728 and 732) differentially expressed in both auxin- and cytokinin-habituated calluses (Fig. 3C). None of these proteins were expressed in control calluses, indicating a specific role in the process of habituation.

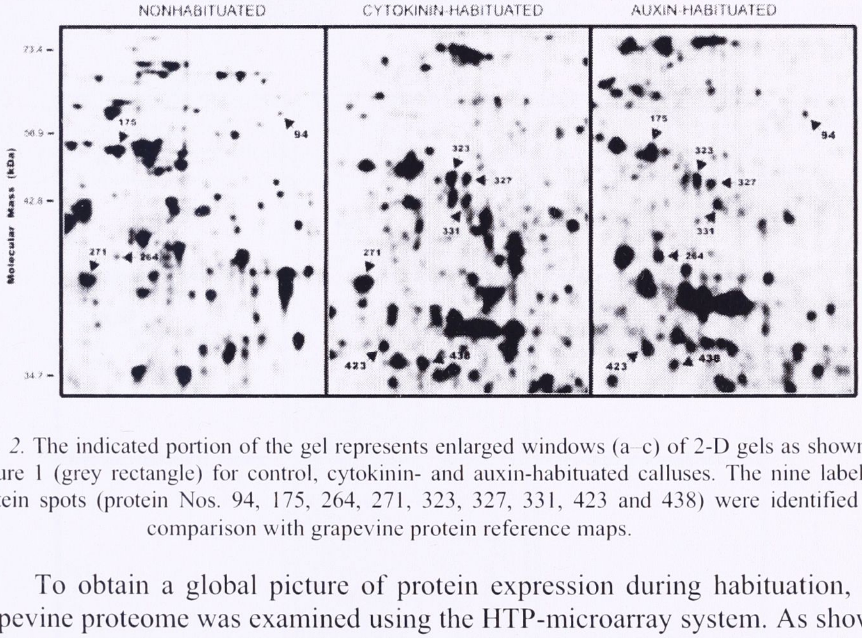

Fig. 2. The indicated portion of the gel represents enlarged windows (a–c) of 2-D gels as shown in Figure 1 (grey rectangle) for control, cytokinin- and auxin-habituated calluses. The nine labelled protein spots (protein Nos. 94, 175, 264, 271, 323, 327, 331, 423 and 438) were identified by comparison with grapevine protein reference maps.

To obtain a global picture of protein expression during habituation, the grapevine proteome was examined using the HTP-microarray system. As shown, both the control and each of the habituated phenotypes had a different, highly specific microarray pattern (Fig. 4A), which corresponded to a different level of expression for the individual proteins in the proteome as the habituation process progressed. As shown in Figure 4B, the expression profile of habituated tissues changed very dynamically. It was observed that 360 d after plating calluses on hormone-free medium the expression profile of both PhA and PhI cultures changed substantially and the proteins expressed were grouped in six clusters, A–F. Functional analysis of these clusters enabled us to discriminate three functional groups based on the positions of individual proteins on the map (Fig. 5). Interestingly, control and/or PhI cytokinin-habituated callus cultures exhibited much smaller overall changes in the proteome profile compared to that of PhA auxin-habituated calluses. These findings illustrated a complex pattern of up- and down-regulation of specific genes in respect to the genetic and metabolic state of the three grapevine callus cultures. Moreover, the results presented here also point to the fact that different gene blocks contribute to diverse extents to the final grapevine proteome profile. Similar observations were described for oxidative stress (Desikan et al., 2001) and brassinosteroid-regulated gene expression in *Arabidopsis* (Goda et al., 2002). A threshold-free functional profiling method was adopted here to analyse the functional classes of proteins "responsible" for the macroscopic changes observed in habituated grapevine callus cultures (Fig. 5). It is evident that functional class 1 is completely uncorrelated with the arrangement, while the other two are clearly associated with high and/or low expression, respectively. The arrow in Figure 5 refers to the multi-functional character of a protein that could belong to more than one functional class.

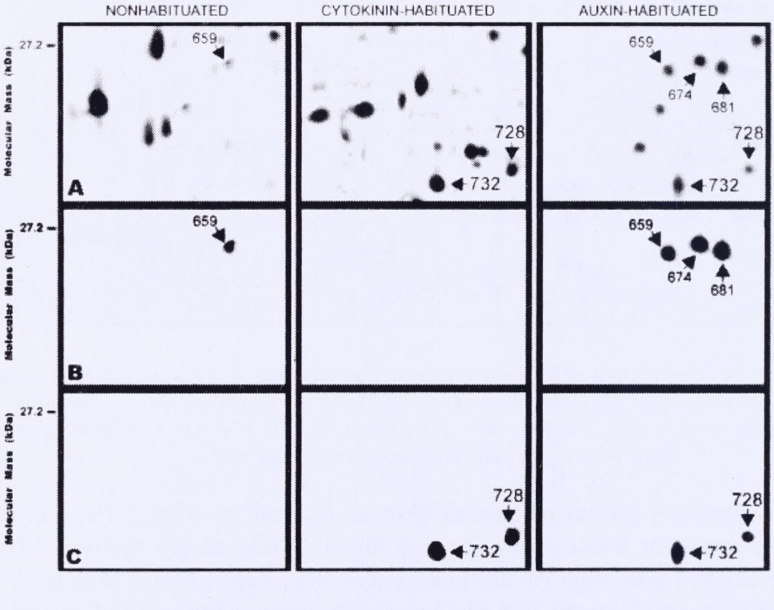

Fig. 3. A. The indicated portion of the gel represents enlarged windows of 2-D gels as shown in Figure 1 (rectangle f). B and C, revelation of specific proteins with the anti-14-3-3 and PRP-10 antibodies, respectively, in the same gel window as shown in A. The five labelled protein spots (protein Nos. 659, 674, 681, 728 and 732) were identified by comparison with grapevine protein reference maps

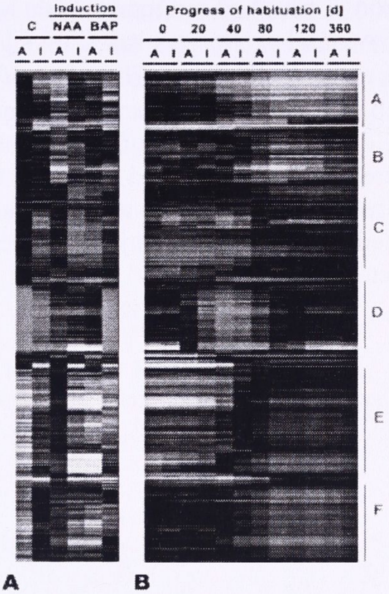

Fig. 4. Expression microarray profiling of non-habituated and habituated grapevine calluses. (A) Heat map shows protein expression levels. (B) Heat map shows the change of protein levels related to the progression of habituation. A – photosynthetically active calluses, I – photosynthetically inactive calluses, C – control. The six (A–F) functional groups of proteins are indicated on the right of the microarray profile

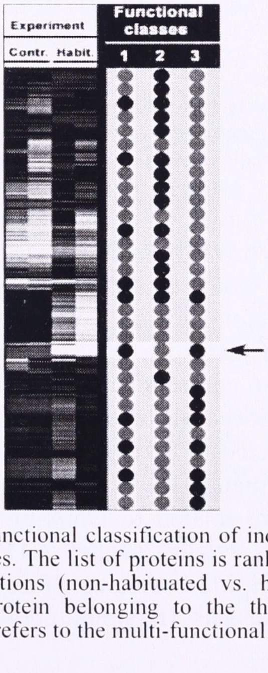

Fig. 5. A threshold-free overall functional classification of individual proteins from control and habituated grapevine callus cultures. The list of proteins is ranked by their differential expression between two experimental conditions (non-habituated vs. habituated). Columns 1, 2 and 3 represent the position of the protein belonging to the three functional classes along the arrangement. The arrow refers to the multi-functional character of the proteins

In the present work the tools of comparative interactomics were used for the biological interpretation of received microarray data sets. The assignment of a spot to a particular behavioural group was based on significant differences in intensity ($P \leq 0.05$) between the replications. These cut-off values served as the basis of the study. Overall patterns of spot changes are summarized in Figure 6. Some proteins showed different patterns of behaviour, depending on the inducing factor, and were therefore assigned to more than one category, as illustrated by the overlaps in Figure 6. For example, a protein may initially exhibit habituation-induced behaviour, but may subsequently be suppressed by somaclonal variability factors.

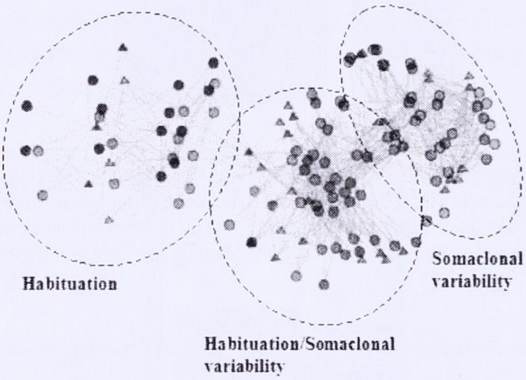

Fig. 6. The Venn diagram represents two interactomes in the protein interaction space. Each portion of the Venn diagram represents the number of spots showing either an increase (↑) or decrease (↓) in spot density. Overlaps contain spots that responded differently to two or more processes (e.g. habituation vs. somaclonal variation).

A structure-oriented protein interaction protocol, PSIMAP (protein structural interactome map, Gong et al., 2001), extracts the exact molecular interaction information of proteins from the Protein Data Bank (PDB) and their domains from the Structural Classification of Proteins (SCOP). Based on protein domain interaction analysis 16 commonly presented protein families were found in the six species. They produced 80 protein family interaction pairs (1.6 links per family), which are predicted to be conserved across all tested species (Fig. 6). Out of the 16 core protein families, 4 (25%) were related to protein translation. Eight (50%) were related to DNA-binding proteins. The last four (25%) protein families were related to the ATP metabolism. These results corroborate previous transcriptome studies (Pischke et al., 2006) on well-conserved and minimal gene sets (Koonin, 2000). The functions of protein families constitute a core network and are mostly related to protein translation, ribosomal structure and biogenesis (Aravind et al., 2000). Overlap analysis (Fig. 6), represented as an interactome network graph, demonstrated that there is a close relationship between habituation and somaclonal variation physiological networks. In fact, using this approach all the proteins of the grapevine proteome were mapped to 12 562 orthologues in the published HTP datasets of yeast, *Drosophila*, worm, *Arabidopsis* and human. This also indicates that the observation of similar interactions in two or more networks increases the likelihood of their biological significance.

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OAT SEED AS AN IMPORTANT SOURCE OF DIETARY FIBRE AND THE INFLUENCE OF GENETIC AND AGRO-ECOLOGICAL FACTORS ON ITS CONTENT

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Among the basic cereals oats (*Avena sativa* L.) are highly valued from the nutritive and dietetic point of view. The chemical constituents of oat seed are therefore in the focus of a number of studies. The effect of genetic and agro-ecological factors on the content of total dietary fibre was investigated in mature grains of ten oat genotypes (5 hulled, 5 naked) grown in two localities during two years. Hulled genotypes contained 33.82% of total dietary fibre in the mature grain, whereas in naked oats, the content of this component was 3 times lower. The effect of the locality was also very significant. Naked genotypes showed a higher content of total dietary fibre in the locality Vígľaš-Pstruša, and hulled genotypes in Borovce. The value of the parameter was significantly influenced by the glumose × locality and year × locality interactions. Levels of total dietary fibre content in hulled oats showed another, highly significant source of variability: the effect of the genotype. Year did not influence the content of dietary fibre in the oat set monitored.

Key words: oats, dietary fibre, locality, year, variability, *Avena sativa*

Introduction

Whole grains became an ingredient of the human diet with the advent of agriculture about 10 000 years ago (Marquart et al., 2002). The positive effects of these seeds and their products on human health have long been known (McKevith, 2004). They are rich in nutrients and phytochemicals with known health benefits.

Among the basic cereals, oats are highly valued from the nutritive and dietetic point of view. In recent years their importance in human consumption has shown an increasing trend. On January 21, 1997, the US Food and Drug Administration (FDA) published a health claim on food-product packages stating that "A diet high in soluble fibre from whole oats (rolled oats, oat bran, oatmeal, and oat flour) and low in saturated fat and cholesterol may reduce the risk of heart disease" (FDA, 1996; 1997). This beneficial effect of oat products is primary attributed to β-D-glucan (FDA, 1997), a component of dietary fibre.

Oats are characterised by high dietary fibre content (Holthaus et al., 1996; Redaelli et al., 2003). The definition of dietary fibre proposed by the American Association of Cereal Chemists (AACC) states that: "Dietary fibre is the edible parts of plants or analogous carbohydrates that are resistant to digestion and absorption in the human small intestine with complete or partial fermentation in the large intestine. Dietary fibre includes polysaccharides, oligosaccharides, lignin, and associated plant substances. It promotes beneficial physiological effects including laxation, and/or blood cholesterol attenuation, and/or blood glucose attenuation" (AACC, 2001). Dietary fibre, in general, shows good effects on dough properties, leading to higher water absorption, mixing tolerance and tenacity, and smaller extensibility. Today there are two reasons to add fibre to baked products: to increase the dietary fibre intake and to decrease the caloric density of foods (Gómez et al., 2003).

The addition of ingredients with significant beneficial health effects or the elimination of ingredients with negative effects on human health is coupled with the preparation of functional foods (Diplock et al., 1999). The potential use of oats in the production of functional foods is associated with the nutritional value of the grain, in particular to the content and composition of protein, lipid and fibre (Redaelli et al., 2003).

Genotype and environment are major determinants of plant phenotype. Economically important quantitative traits include agronomic characteristics and grain composition. To understand which factors affect the value of dietary fibre in mature grain and how is important for more effective raw material cultivation and for their utilization in the industry. The present results will also be the starting point for further studies which will consider oats as a beneficial material for the food industry.

Materials and methods

Seed samples of ten oat genotypes (naked – Avenuda, Detvan, Izak, PS-90, Salomon, and hulled – Edit, Edmund, Expander, Petra, Zvolen) harvested in two years (2006, 2007) in two localities (Borovce and Vígľaš-Pstruša) were used in this study. Each variety was grown in three different plots of 2.5 m^2 in a fully randomised block design.

The soil type in Borovce is loamy luvic chernozem. It is a transitive maize–sugar beet growing region with a mean annual temperature of about 9.2°C (15.5 °C for growing season), a mean annual precipitation of 593 mm (of which, 358 mm in the growing season), and an elevation of 160–180 m. In Vígľaš-Pstruša, a potato-growing region, the soil type is podzolic brown, the mean annual temperature 8.0°C, the mean annual precipitation 666 mm, and the elevation 375 m.

Mature grains were milled to pass through a 0.5 mm sieve using an Ultracentrifugal Mill (ZM 100 Retsch, Germany). The level of total dietary fibre was determined using the total dietary fibre assay procedure (Megazyme, Ireland). This method is based on the methods of Lee et al. (1992) and Prosky et al. (1988; 1992) and accepted by the AOAC (Method 991.43, 985.29) and the AACC (Method 32-07, 32-05) (McCleary, 2007). Total dietary fibre (TDF) was determined on duplicate samples of dried and defatted (if fat content is >10 %) material. The samples were cooked at ~100°C with heat-stable α-amylase to achieve the gelatinisation, hydrolysis and depolymerisation of the starch, then incubated at 60°C with protease (to solubilise and depolymerise proteins) and amyloglucosidase (to hydrolyse starch fragments to glucose) and treated with four volumes of ethanol to precipitate soluble fibre and remove depolymerised protein and glucose (from the starch). The residue was filtered, washed with 78% ethanol, 95% ethanol and acetone, dried and weighed. One duplicate was analysed for protein and the other was incubated at 525°C to determine the ash content. The TDF was the weight of the filtered and dried residue less the weight of the protein and ash. The nitrogen level was determined using the Dumas method (CNS-2000 Elemental Analyzer, LECO Corp., USA) and the content of crude protein using a factor of N × 6.25.

The general linear model (GLM), analysis of variance and Bonferroni test were carried out using the SPSS for Windows (Release 11.5.1.) program.

Results

The TDF content in naked oats ranged between 11.9% (PS-90) and 13.0% (Detvan), with a mean value of 12.4%. In hulled genotypes, the mean value of this parameter was 33.82%, which was 3 times higher than in naked genotypes. It can thus be stated that the TDF content was highly significantly influenced by the presence of glumes ($p<0.01$). The Tukey HSD test showed naked oat genotypes to form one uniform group; no differences were found between the mean levels (data not shown). The influence of genotype as a source of variability was significant in the hulled oat set, where a considerable range of variation was observed (Table 1), from 30.1% (Edmund) to 36.2% (Expander). The TDF content rose in the following order: PS-90 < Avenuda < Salomon < Izak < Detvan < Edmund < Edit < Petra < Zvolen < Expander. The last three genotypes (Expander, Zvolen and Petra) had TDF contents above the mean value and are therefore suitable natural sources of total dietary fibre.

The effect of locality on the value of the monitored parameter was notable. GLM indicated the highly significant influence of the growing locality ($p<0.01$) in both the analysed groups. The TDF content in naked oats was significantly higher in Vígľaš-Pstruša (13.3%) than in Borovce (11.5%). In hulled genotypes the opposite was observed. The mean fibre content was higher in Borovce (36.4%) than in Vígľaš-Pstruša (31.3%).

The influence of locality and year on the TDF content in mature oat seeds was also investigated. Statistics showed a highly significant influence of locality and highly significant influence of year × locality on the TDF content in naked oats. For hulled oat genotypes, the highly significant effect of the variety was recorded. Both groups (naked and hulled) gave a slight response to the cultivation year, but no significant differences in the level of TDF were shown.

According to these results, the genotype × growing locality interaction was not statistically significant in naked oat genotypes. Investigations on the higher plasticity of the naked variety PS-90 produced interesting results. The effect of locality (average 11.8% in Borovce and 12.1% in Vígľaš-Pstruša) was less noticeable in this case. In other naked oat genotypes, the TDF content was more distinctly influenced by the locality; in particular the average content was higher in Vígľaš-Pstruša.

Table 1

Mean squares (MS) from the analysis of variance for the total dietary fibre content of a set of oat genotypes

Source of variability	df	MS	
		Naked oats	Hulled oats
Model	20	462.211**	3484.475**
Genotype	4	2.015	76.745**
Year	1	1.104	0.007
Locality	1	47.313**	386.436**
Genotype × year	4	1.297	6.813
Genotype × locality	4	2.229	6.967
Year × locality	1	20.862**	316.159**
Genotype × year × locality	4	3.812	2.187
Error	40	2.319	6.596
Total	60		

** $p<0.01$ (effect significant at the $\alpha = 0.01$ level)

The highly significant ($p<0.01$) year × locality interaction for naked oat seeds was almost entirely due to differences in the magnitude of TDF values in the two environments analysed. In the year 2006 it was 13.7% in Vígľaš-Pstruša and only 10.7%. in Borovce Equally, in 2007 the average content of TDF was 12.8% in Vígľaš-Pstruša and 12.2% in Borovce.

In both groups of genotypes the year × locality interaction was highly significant. The effect of the year 2007 was mostly observed in Vígľaš-Pstruša, where the mean value of TDF in hulled oat seeds decreased sharply to 29.0% in comparison with 2006, when the average value was 33.6%. It was also observed that the difference between the two localities was more marked in 2007 than in 2006. On the other hand, in naked oat seeds grown in Vígľaš-Pstruša in 2006 the mean value of TDF was much higher than in 2007.

The genotype × year interaction was not important in either naked or hulled oat varieties. Although differences were observed in the TDF content between individual genotypes, they were non-significant.

The results for the genotype × year × locality interaction in naked varieties were diametrically opposite. In Borovce the seed samples gave different respondses to the year. In 2006 the TDF content was lower than in 2007, with the exception of the variety Salomon (Table 2), where it was higher in 2006 (11.4%) and lower (10.9%) in the second year. The smallest differences were observed in the genotype Detvan and the greatest in Izak and Avenuda. In Vígľaš-Pstruša the TDF content was higher in 2006 than in 2007, except for Salomon (13.1% in 2006 and 13.8% in 2007). The decrease in fibre was clear in all the naked genotypes in 2007, with losses of 1.4 % in Avenuda 1.7% in Detvan, and 1.4% in Izak.

Table 2

Mean values of the total dietary fibre content (TDF) in naked oat genotypes as % dry matter

Genotype	Year	Locality	Mean TDF (%)	Standard deviation
Avenuda	2006	Borovce	10.0	1.6
		Vígľaš-Pstruša	13.6	1.1
	2007	Borovce	12.5	0.5
		Vígľaš-Pstruša	12.2	2.5
Detvan	2006	Borovce	11.9	1.2
		Vígľaš-Pstruša	14.8	1.6
	2007	Borovce	12.0	0.9
		Vígľaš-Pstruša	13.1	1.3
Izak	2006	Borovce	9.6	0.5
		Vígľaš-Pstruša	14.5	1.5
	2007	Borovce	12.9	2.1
		Vígľaš-Pstruša	13.0	2.0
PS-90	2006	Borovce	10.8	0.3
		Vígľaš-Pstruša	12.4	1.0
	2007	Borovce	12.7	1.7
		Vígľaš-Pstruša	11.8	2.1
Salomon	2006	Borovce	11.4	0.5
		Vígľaš-Pstruša	13.1	1.5
	2007	Borovce	10.9	1.3
		Vígľaš-Pstruša	13.6	2.5

In hulled genotypes the genotype × year × locality interaction was not significant. The mean values (Table 3) ranged from 27.8% (Edmund) to 36.6% (Expander) in Borovce in 2006 and from 35.1% to 42.0% in 2007. In Vígľaš-Pstruša the values ranged from 30.0% (Edmund) to 36.4% (Expander) in 2006 and from 27.4% (Edit) to 31.8% (Zvolen) in 2007.

Discussion

The results indicating a higher content of TDF in hulled oat genotypes are similar to those reported by Grausgruber et al. (2004), who observed a mean TDF content of 14.7% in naked oat genotypes and 41.6% in hulled. It is clear from the results that the glumes are the part of the cereal grain where the highest level of fibre components is concentrated.

It can be concluded that the TDF content in naked oats was more constant in Vígľaš-Pstruša, with the fibre content being more strongly influenced by the environment in some cultivars (Edit, Petra, Salamon, Izak) than in others (Zvolen, PS-90). The latter had a more consistent response to the environment. This could be due to the presence of glumes. Grains of naked genotypes are not so well protected by glumes as hulled, so they are more sensitive to dry conditions, where they lose moisture. Therefore, it can be predicted that their cultivation will be more effective in localities with lower mean temperature and higher humidity.

Table 3

Mean values of the total dietary fibre content (TDF) in hulled oat genotypes as % dry matter

Genotype	Year	Locality	Mean TDF (%)	Standard deviation
Edit	2006	Borovce	33.7	1.8
		Vígľaš-Pstruša	32.0	1.7
	2007	Borovce	36.9	3.3
		Vígľaš-Pstruša	27.4	0.1
Edmund	2006	Borovce	27.8	1.3
		Vígľaš-Pstruša	29.9	1.0
	2007	Borovce	35.1	2.6
		Vígľaš-Pstruša	27.6	1.2
Expander	2006	Borovce	36.6	3.6
		Vígľaš-Pstruša	36.4	4.7
	2007	Borovce	41.9	2.8
		Vígľaš-Pstruša	30.0	1.3
Petra	2006	Borovce	36.1	2.4
		Vígľaš-Pstruša	34.3	1.3
	2007	Borovce	39.3	0.9
		Vígľaš-Pstruša	28.2	1.9
Zvolen	2006	Borovce	36.1	3.0
		Vígľaš-Pstruša	35.3	1.7
	2007	Borovce	40.1	2.9
		Vígľaš-Pstruša	31.8	5.2

A significant correlation (r=–0.90**) was observed between TDF content and the content of β-D-glucan in oat seeds (Havrlentova et al., 2008). Peterson et al. (2005) observed significant genotypic differences in the content of β-glucan, a component of dietary fibre. Statistically significant differences were caused by the growing conditions. Among the agronomic markers, grain yield was most influenced by the environment, while the amount of β-glucan was significantly affected by the genotype (p=0.01). The concentration of polysaccharides also fluctuated in response to the genotype × year interaction. Yalçin et al. (2007) studied the effect of variety and environment on the TDF and β-D-glucan content in naked barley genotypes and found significant differences according to variety and growing locality. Significant correlations were observed between the TDF content and the thousand-grain weight, hectolitre weight and yield. Torp et al. (1981), on the other hand, detected a negative relationship between the fibre content in mature barley seeds and the yield.

A non-significant genotype × year interaction was shown in the present work and also in that of Manthey et al. (1999), while Sgrulletta et al. (2004) detected significant differences in the dietary fibre content according to genotype and environmental conditions during growth. There were highly significant effects for variety, locality and their bilateral interaction, but the highest effect was observed for the genotype (Zhang et al., 2002). Similar results were reported by Pérez-Vendrell et al. (1996), who noted that the β-D-glucan content was higher in winter varieties of barley than in spring.

The TDF content in hulled oats in Borovce appears to have been influenced by the precipitation deficit in summer (July 2006) and autumn (September 2006), which continued at the beginning of the next season (April 2007). The reason could be that drought negatively influenced grain production, causing a higher glume fraction, resulting in a higher content of dietary fibre. Analogous results were published when the influence of agro-ecological factors on the β-D-glucan content was analysed by Cho and Dreher (2001), who found the greatest effect for the locality, often combined with that of climatic conditions and soil type. As reported by Peterson et al. (1995), long-term drought stimulated the β-D-glucan accumulation in oats. Precipitation in July and August had no influence on this parameter (Cho and Dreher, 2001).

In hulled oats no statistically significant genotype × locality interaction was found. This was in accordance with the work of Aalto et al. (1988), who studied the content of total, soluble and insoluble dietary fibre in Finnish barley genotypes.

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PRELIMINARY REPORT ON THE USE OF BIOTECHNOLOGY IN SWEET AND SOUR CHERRY RESEARCH

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The goal of this investigation was to determine genetic differences between autochthonous and introduced cultivars of sweet cherry and between cultivars and types of sour cherry, and to find and optimize a method for the rapid recovery of clonal material. A great number of cherry cultivars and types within the population of cv. Oblačinska sour cherry exist in Croatia and the selection of autochthonous cultivars based on special visible properties for further investigation has been done in previous research. Differences were found in a number of important agronomic traits within the populations of cv. Oblacinska sour cherry. It is suspected that autochthonous sweet cherry cultivars are synonyms for known old cultivars, which were introduced randomly and naturalized under local names. In this approach difficulties arise from the effect of non-genetic factors on the expression of certain traits. The genetic/physiological problem of S allele autoincompatibility exists within sweet cherry cultivars. The detection of S alleles is required to determine compatible cultivar pairs in the orchard. Biotechnological methods based on the polymerase chain reaction (PCR) facilitate faster virus detection compared with classical serological methods and indexing. Thermotherapy and tissue culture make it possible to recover valuable clone material for introduction in the premultiplication process.

Key words: sour cherry, sweet cherry, clonal selection, autochthonous cultivars, thermotherapy, tissue culture

Introduction

Cv. Oblačinska sour cherry is a leading cherry cultivar for the processing industry in Croatia because of its pomological characteristics, suitability for mechanical harvesting, earliness and good fertility. Although sour cherry is vegetatively propagated (all progeny have the same parental genotype), intracultivar variation has been noted in existing productive plantations in Croatia, implying clonal selection within the population. Intracultivar variability is the result of generative propagation and mutations caused by natural mutagenic factors.

Investigations on morphological, pomological and chemical characteristics have been made on 42 divergent types, multiplied by grafting on CAB 6P rootstock. A collection of 42 clones from Croatia, five from Serbia, three clones of cv. Maraska and three standard cultivars: Heimans Konservenweichsell, Kelleris 14 and Rexelle, were studied with microsatellite markers (SSR – Simple Sequence Repeats). The results obtained for eight microsatellite loci (Cipriani et al., 1999; Dirlewanger et al. 2002; Wünsch and Hormaza, 2002) showed no variability between clones of cv. Oblačinska, but they clearly demonstrated differences in relation to other cultivars: Maraska, Heimans Konservenweichsell, Kelleris 14 and Rexelle (Puškar, 2005).

Viruses such as prune dwarf virus (PDV) and sharka or plum pox virus (PPV) can be detected and identified with serological methods (DAS-ELISA) and molecular methods (RT-PCR) (Youssef et al., 2002; Sertkaya et al., 2003). Plant material treated with thermotherapy *in vitro* and propagated by axillary shoot production (meristem culture) is free of viruses and can be rapidly multiplied with micropropagation techniques (Cerović and Ružić, 1987; Laimer et al., 2006).

Our goal was to test the hypothesis that cv. Oblačinska is polyclonal, i.e. a population of different genotypes in a planted experimental orchard that contains selected clones of cv. Oblačinska in the homogeneous state. It is necessary to establish a key for the identification of selected clones of cv. Oblačinska according to morphological and genotypic markers. An analysis of the health status of selected clones of cv. Oblačinska according to EPPO standards will be necessary for obtaining healthy prebasic material. The material obtained will be treated with thermotherapy and micropropagated by meristem culture *in vitro*.

Materials and methods

A collection of 42 clones from Croatia, five from Serbia, three clones of cv. Maraska, three clones of a population of Cigánymeggy from Hungary and three standard cultivars: Heimans Konservenweichsell, Kelleris 14 and Rexelle, were grafted onto the *Prunus mahaleb* rootstock and planted in the orchard of the Agricultural Institute Osijek in a randomized block design with three trees per block and four replications, giving 12 trees of each type/clone. The trees were grown under uniform conditions for a more objective evaluation. Three types/clones will be selected for further investigation, involving thermotherapy and micropropagation.

For genotype identification DNA was extracted from lyophilised leaf tissues of sour and sweet cherry using a mi-Plant Genomic DNA Isolation Kit (Metabion, Germany). PCR reactions for eight different SSR primer combinations (Cipriani et al., 1999; Dirlewanger et al. 2002; Wünsch and Hormaza, 2002) were performed in 25 μl volumes containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2 mM $MgCl_2$, 0.2 mM dNTP, 0.4 μM each primer, 40 ng genomic DNA and 1 U of Taq polymerase. The PCR cycle conditions consisted of an initial step of 2 min at 94°C, 35 cycles of 45 s at 94°C, 45 s at 59°C, 1 min at 72°C, and a final step of 5 min at 72°C.

The PCR products were separated by electrophoresis using 4% agarose gels in 1× TBE buffer at 90 V. Microsatellite markers can detect differences between cultivars, but for clonal identification AFLP markers will be used (Vos et al., 1995; Struss et al., 2001). After clonal selection by AFLP, selected material will be tested for virus contamination using serological

(DAS-ELISA) (Youssef et al., 2002; Sertkaya et al., 2003) and molecular methods (RT-PCR) to verify and identify the viruses (Youssef et al., 2002; Sertkaya et al., 2003). Before the propagation of meristem cultures the infected material will be subjected to thermotherapy (Cerović and Ružić, 1987; Laimer et al., 2006).

Results

So far, microsatellite marker analysis (SSRs) has shown differences between cultivars (Maraska, Kereška, Oblačinska) but not between clones/types of cv. Oblačinska.

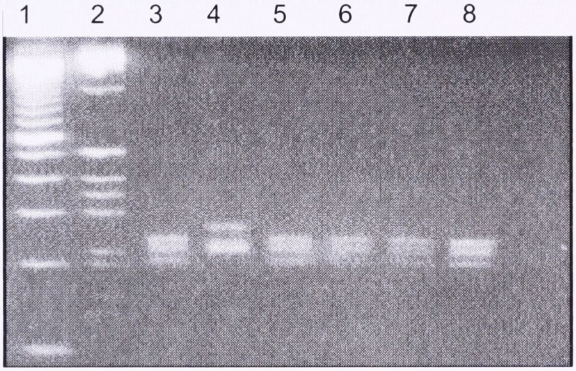

Fig. 1. Electrophoregram showing the presence of microsatellite locus BPPCT-034 in sour cherry cultivars: 1. DNA Molecular Weight Marker XIV (100 bp ladder), Roche, 2. DNA Molecular Weight Marker X, Roche, 3. Maraska, 4. Kereška, 5., 6., 7. and 8. Sour cherry cv. Oblačinska types/clones

The first experimental *in vitro* laboratory for micropropagation was established in the Agricultural Institute Osijek in 2006. Protocols were established for the micropropagation of sour cherry from axillary shoot production (meristem culture) and for thermotherapy.

This paper presents the research plan and results achieved so far in the project entitled Biotechnological methods in identification, selection and propagation of sour cherry (*Prunus cerasus* L.). It is hoped that this project will result in the first virus-free prebasic material of cv. Oblačinska clones with good pomological characteristics. The research will then be extended to other fruit species to improve commercial orchards in Croatia. The micropropagation now in progress is the first step in modern tree production.

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REMARKS ON THE CURRENT DISCUSSION ABOUT BIOENERGY – FOR THE PUBLIC OR FOR AGRICULTURAL AND RURAL AREAS ONLY?

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In this paper, strategies are questioned and it is discussed whether the goals of the EU commission to replace substantial parts of the fossil energy demands by bioenergy supplies are feasible. Austria, as a member of the PPBA (Pannonian Plant Biotechnology Association), has elaborated a study on how much of the arable land can be utilized in the period between 2005 and 2020 for various bioenergy purposes. The results demonstrate that, at the most, agriculture can supply only about 22% of the total arable land needed for additional bioenergy, such as biofuel and biogas, without interfering with national food and feed supplies, and the protection of a sensible situation as regards the environment and emission.

Furthermore, two research studies are presented about new production systems to achieve the better, more efficient use of crop rotations, mainly for biogas production, already being implemented in the 358 local farm biogas plants in Austria, and about the approved long-term use of the whole plant biomass of triticale varieties for heating local private and farm houses.

Key words: bioenergy, energy crops, biofuel, biogas, biomass

The production of agroenergy from biomass should be performed in a system well balanced between areas cultivated for food and feed and areas used for energy crops. All research efforts and investments must have the goal of utilizing the whole plants for energy in order to improve the relatively low energy content of the various plant parts. Based on the present status of technology for biomass conversion into energy, the OECD has calculated that in the USA 30% of all acreage of cereals, sugar beets and oil crops could only replace 10% of the present total petrol consumption. For Europe, 50% of the respective crop areas are not enough to replace 10% of the demand for fossil fuels. Probably the next generation of fuels, known as BtL fuels (Biomass to Liquid), will change this high demand worldwide for agricultural areas

(Anonymous, 2007). Worldwide programmes to replace significant proportions of the present fossil energy carriers by renewable bioenergy are focused on bioethanol and biodiesel, but the amount of plant and crop resources that can be converted into liquid fuel without interfering with the protection of ecosystems and with worldwide nutrition needs is very limited. It is questionable whether the estimated global net primary production capacity of 50 to 60 Gigatons of renewable biological carbon per year can satisfy the estimated future consumption of about 45 Gigatons per year by mankind for food, feed, chemicals and energy in 2050. The present situation in many areas of the world, where renewable plant resources for agroenergy are being exploited on newly cultivated arable land with monocultures of energy plants at the expense of forests and grassland, is going in the wrong direction. Serious discussions and arguments are underway about the upcoming competition between food and energy production within global agriculture. The fast-rising prices recently reported for wheat and maize and the outcry of the consumers on both sides of the Atlantic demand a completely redesigned strategy. The future goals of producing food, feed and raw materials for chemicals and different forms of energy must inevitably be based on the limited arable land already present. It must unquestionably be utilized in a sustainable manner to preserve durable soil fertility and to meet environmental requirements. Due to the fast-rising prices for small grain cereals and maize in 2007 and in the first months of 2008, and the appearance of critical food shortages, China stopped all further projects to produce bio-ethanol from cereals and maize in autumn 2007 (Liu, 2006). China, with its high population, was the first country to take this serious decision, a decision which is being given more and more consideration in many other parts of the world. Nevertheless, on the other side of the globe, in Brazil and in the USA, the trend to produce more bioethanol seems to be continuing. In 2002 both countries produced approx. 20 billion litres, rising to 35 billion litres in 2006, with an estimated goal of increasing production further to approx. 50 billion litres by 2012. In comparison with these figures, the production capacity of Canada, China, India and the EU did not even reach the 2 billion level in 2006 (Anonymous, 2008).

Based on the efficiency with which ethanol can be produced from agricultural crops, sugar cane is without any doubt a far better option than maize or wheat. The trend to produce expensive bioenergy from non-replaceable food and/or feed crops is extremely questionable. This discussion is also necessary in Europe, because the production figures for bioethanol increased steadily from 2004 to 2007. The estimated production figures of about 1.77 billion litres in the EU, with France as the main producer, are aimed to be doubled in 2010. This is likely to reduce the capacity for food and feed production. For example, France forecasts the use of 7.5% of its wheat and 19% of its sugar beet production for an ethanol capacity of about 1.4 billion litres (66% from wheat, 19% from sugar beet). The estimated 20% share of total arable land used for bioenergy purposes in EU member countries will not be sufficient alone to meet the high expectations of replacing 20% of fossil fuel by bioethanol or biodiesel. However, the EU bioethanol fuel sector has constantly been confronted with very high prices for wheat and maize. Therefore, the price of raw material costs in bioethanol production went up to a level that made production no longer profitable. So some companies decided either to stop operating temporarily or delayed the construction of new plants.

On a smaller scale this scenario is also present in Central Europe. A recently published survey from the Austrian Bio Energy Centre (Anonymous, 2008) demonstrates a sharp increase in existing capacities in this region between 2006 and 2008, with eight fuel plants at present operating in Austria, the Czech Republic, Hungary and Slovakia. However, one Austrian plant, constructed in Pischelsdorf, Lower Austria, with a planned capacity of 200 million litres was opened in summer 2007 but not started up because of the high price of raw materials. The reason for this disaster, involving a 40 million Euro investment, was that calculations were based on a wheat price of 90 € per ton, which has now increased steeply to 220 €. The fate of other enterprises in the EU and in other countries in Europe is still in doubt, demonstrating the uncertainty of the changing situation in this field. A similar situation is likely to dash euphoric hopes for biodiesel. Water supplies, crop rotation problems and high input costs for the production of oilseed rape and sunflowers will limit the supply of this raw material for cost-efficient economical production.

The situation outlined above raises the need for an economic, environmentally sound strategy for national and global decisions:

1. How much arable land can be utilized for agroenergy without interfering with future supplies of food and feed for the national economies?
2. How can agricultural systems contribute substantially to reducing the amount of fossil energy consumed in order to lower the toxic emissions that are endangering the global climate?

Question 1

To face this situation realistically, national situations can be expected to dominate all future concepts of how much agroenergy agriculture as a whole can contribute for public use or for rural areas only. Investigations in Austria (Spitzer et al., 2007) demonstrate that in 2005 only 5% of the arable land was used for bioenergy (Tables 1 and 2). Oilseed rape and sunflower contributed exclusively to the low level of biodiesel production. Biogas was produced by about 295 on-farm plants, the raw material of which was mainly maize silage mixed with liquid cattle manure. The technology for producing biogas on farms is fairly well developed and now bigger farm communities do not hesitate to invest in this field for local energy supplies. Based on a careful investigation, this picture of Austrian agriculture is likely to change considerably in the next 12 years. A nearly threefold rise in the oil crop area for biodiesel will be accompanied by a dramatic increase in starch- and sugar-containing crops from 1 230 ha to 43 000 hectares. This will be achieved through a decrease in the fodder crop area due to reduced animal production and to progress in efficiency, leading to higher energy output than can be reached at present. The genetic improvement of varieties, resulting in about 1.5% yield gain per year, will strengthen the shift towards energy plants in all arable crop production systems. The bioenergy industry will be based mainly on wheat for bioethanol and on maize silage, produced from specific maize varieties, for the production of biogas. Due to a loss of about 15 hectares of arable land a day for settlements, roads, railway tracks and public areas, the total arable land in Austria will steadily decrease to 1 296 975 ha in 2020. However, about 21.7% of this reduced land area will probably be used exclusively for bioenergy. This is in line with predictions for other EU countries. The promising development of the dry combustion of lignocellulose-containing plant materials from residues such as straw or hay to produce BtL (Biomass to Liquid) biofuel via the Fischer-Tropsch process will also contribute to the bioenergy supply, as shown in Table 2. By comparing the situation for 2005 with estimations for 2020, the utilization of the cereal straw harvest from about 42 500 ha will contribute significantly to future bioethanol production. This development should not be overestimated; the energy gain from agricultural crops is still rather low and in many cases their contribution to agroenergy is greatly overestimated.

Table 1

Estimation of arable areas available for bioenergy in Austria in the period 2005–2020 (in 1000 hectares) (Spitzer et al., 2007)

Group of energy plants	2005			2020		
	Biofuel	Biogas	Total	Biofuel	Biogas	Total
Oil crops (rape seed, sunflower, others)	17.4	0.80	18.20	47	4	51
Starch/sugar crops (cereals, maize, sugar beet)	–	1.23	1.23	33	10	43
Energy crops (*Mischantus,* cereals, maize)	0.52	58.71	59.23	66.5	121.5	188
Total agroenergy area	17.92	60.74	78.66	146.5	135.5	282
Arable land/% share	1,379,100		5.7%	1,296,975		21.7%

Table 2

Estimation of arable land crop residues for additional agroenergy use in Austria from 2005 to 2020 (in 1000 hectares) (Spitzer et al., 2007)

Areas/crop residues	2005			2020		
	Biofuel	Biogas	Total	Biofuel	Biogas	Total
Cereal straw	–	1.8	1.8	40	2.5	42.5
Maize straw	–	–	–	5	0.1	5.1
Others	–	–	–	3	0.5	3.5
Additional areas for agroenergy supply	–	1.8	1.8	48	3.1	51.1
Percentage of total arable land		0.13%			3.94%	

Question 2

Several options can be offered, starting from small-scale changes for local farm communities up to national programmes for the public in certain situations. Many international studies show that all the useful kinds of biomass give the highest energy yield and the greatest reduction in emissions when used for heating and electrical power. So the debate on the exploitation of biomass for biofuel and/or biogas is still open and needs a well-balanced decision. There is no doubt that a reduction in fossil fuel consumption should be the primary goal. For a transitional period of 10–15 years a mixture of soil gas (80%) and biogas (20%) could be a possible option. Engines driven by this highly concentrated methane mixture run smoothly, with a reduction of about 25% in emissions compared with petrol-driven engines. One unresolved problem is the elimination of CO_2 from the biogas to achieve a methane concentration of about 97%. The rapidly rising prices for CO_2 emission payments, in accordance with the Kyoto protocol, will undoubtedly force research in this direction. The results of a study in Austria demonstrate that the exploitation of the biogas produced by already existing biogas plants as transport fuel could lead to a reduction of about 70% in the present 22 million tons of CO_2 emissions in this country. However, there are possibilities for agricultural production to contribute to energy solutions for the future. An intelligent combination of various conversion technologies could broadly achieve a balance between food/feed production and the bioenergy supply. Two examples will be presented:

Example 1

Long-term field experiments in Austria (Amon et al., 2007), performed at the University for Natural Resources and Life Sciences in Vienna, will offer a possible solution in this direction (Table 3). In a 5-year crop rotation experiment with maize, winter wheat, intercrop clover, sugarbeet, sunflowers and alfafa, the food and feed production gives enough capacity for an average fuel production of 0.5 tons of crude oil units (ethanol) per hectare. Over the five years only two grain harvests, those of wheat and maize, are used for fuel energy, whereas four times this amount, 2 tons of crude oil units per year in the form of biogas, is gained from the other crops. The average annual gain could theoretically achieve a production of 2.5 crude oil units per hectare and year. In cases where the straw residue from wheat and maize can be prepared for biogas production by steam treatment techniques, this figure could reach an annual energy yield level of about 2.7 tons of crude oil units from maize straw or 2.7 tons of biomass from sunflower crop residues. This could be feasible if new harvesting techniques for both crops were available (Table 3, figures in brackets). A theoretical transfer of this situation to the 93 million hectare arable land area of the EU could then produce 215 million tons of crude oil energy units. The total fuel demand for road transport is about 334 million tons in the EU alone. Comparing these two figures, a substantial amount of the energy demand could be supplied using the above or any other modified sustainable crop rotation system.

Table 3
Biogas energy from agricultural crops – results of a 5-year crop rotation trial (Amon et al., 2007*)*

Year	Crop	Energy yields in crude oil units/ha/year
1	Maize grain (ethanol)	1.535
	Maize stillage (35% d.m.)	1.022
	Maize straw	1.287 (2.736)
2	Winter wheat grain (ethanol)	0.912
	Winter wheat stillage (40% d.m)	0.608
	Winter wheat straw	0.616 (1.311)
	Intercrop clover	0.862
3	Spring barley straw	0.520
4	Sugar beet leaves (fresh)	0.520
	Sugar beet silage	1.093
5	Sunflower straw	1.444 (2.693)
	Intercrop alfalfa	1.149

Average ethanol yield/rotation/year	0.489
Average methane yield/rotation/year	2.008
Average energy yield/rotation/year	2.497

Example 2

The production of solid fuel from agricultural crops, e.g. cereal grains and straw, using a whole plant harvesting technique, was already started 15 years ago. In Wolfsthal, Lower Austria, energy for heating and hot water for about 400 family homes and 85 farms was supplied by burning whole plant cereal crops in the form of hard pressed bales in a heat power station near the village (Ruckenbauer et al., 1996). The preliminary field experiments for the establishment of a local energy supply at low cost clearly showed the high yield potential of triticale for biomass production aimed at energy utilization. The yields reached more than 12 tons of biomass per hectare under intensive farm management with moderate fertilization and plant protection (Table 4). Even under non-intensive farm management (only fertilizers, no herbicides or pest control) and on less fertile soils, about 9 tons per hectare 'whole cereal plant biomass' (whole plants harvested at the milky ripe stage of the crop, after field drying and specific bale pressing techniques) was produced. Investigations to compare variety differences and/or differences between plant biomass and the firewood hitherto used demonstrated near equality between the energy values of the whole plant material of the varieties investigated and the common firewood used as a control (Table 5). Therefore, the annual production of about 2–3 ha triticale at each of the 85 farms could supply enough heating energy for the whole community throughout the year, for costs of about two thirds of the usual energy prices paid during the period of 14 years. This situation was undoubtedly favoured by the low transport costs of the energy source from nearby field areas and the lack of wood fuel sources in this eastern part of Austria.

Table 4
Whole plant yields at different production levels in solid fuel trials
(Wolfsthal, Lower Austria, 1993–1994; Ruckenbauer and Reichert, 1996)

Whole plant material Varieties/Species	Quintals/hectare			Range of yields	
	Intensive	Non-intensive	Mean	Minimum	Maximum
Almo/triticale	112.0	105.2	108.6	90.7	124.6
Hai/triticale	103.3	94.1	96.7	79.1	115.4
Capo/Winter wheat	100.7	91.6	96.2	76.6	112.1
$LSD_{5\%}$	4.85		6.87	Mean 1993: 89.9	
				Mean 1994: 107	

Table 5
Energy values – comparison between whole plant material from triticale, winter wheat and firewood (Ruckenbauer and Reichert, 1996)

Plant energy sources (intensity levels)	Water content (%)	Ash content (%)	Energy efficiency (%)	Energy values (MJ/kg)
Almo/triticale				
Intensive	13.0	14.9	77.6	14.37
Non-intensive	12.4	5.0	78.1	14.50
Capo/winter wheat				
Intensive	11.8	5.5	77.3	14.86
Non-intensive	12.7	4.8	77.6	14.64
Firewood (control)	13.2	0.8	82.0	15.90

At present many options are under discussion as to how different national agricultural and management systems could contribute to an environmentally sound energy supply. However, at present, there are high food prices and rising energy demand worldwide and the difficult question of food vs. fuel will not be easy to answer.

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FOOD VS. FUEL – A TURNING POINT FOR BIOETHANOL?

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Bioethanol is made from sugar- or starch-containing plants that are also used in food production. In the public perception this has led to an emotional resistance against biofuels, which in real terms is not substantiated. Generally biofuels are a political product. Triggered by the oil crisis in the 1970s, fuel ethanol programmes were first launched in Brazil and in the United States. Concerns regarding energy security and sustainability, together with the option of new markets for surplus agricultural production, have led to similar measures in the EU and other countries in recent years. Accordingly, the industry invested heavily in new bioethanol plants – especially in the US – and created an additional demand for maize and wheat, with some record-breaking prices noted in late 2007. A look back into statistics shows a drastic decline in real prices for decades, which have now simply returned to the level of 30 years ago. The grain used for bioethanol is currently only 1.6% in the EU and is therefore unlikely to be the real driver of price development. The European Commission concludes in its review of agricultural markets that Europe can do both: nutrition and biofuels.

Key words: bioethanol, fuel ethanol, food versus fuel

Introduction

Ethanol marked the beginning of automotive fuels. It was already considered as a source of energy by Nicolaus August Otto and Henry Ford, whose *Ford Model T* ran on ethanol, petrol and any combination of the two fuels – what we call now a flexible fuel vehicle. Cheap fossil fuels, however, dominated the market after World War II and it was only during the oil crisis in the 1970s that governments began to think about alternatives.

Key stake-holders

The first country to launch a national fuel ethanol programme was Brazil. The *Proalcool* programme envisaged deriving ethanol from locally grown sugar cane and using it instead of petrol. This required modified engines, but the ethanol price was so attractive that after a while 2.5 million cars ran on pure alcohol. Later, high sugar prices on the world market affected production and the consumption dropped gradually. Today there is a renewed boost for bioethanol with a considerable price advantage over petrol. All filling stations offer blends with 25% ethanol (this value is set by the government, depending on the sugar market). The major part of car sales are now FFVs (flex fuel vehicles) with modified combustion engines for any mix of petrol and ethanol up to 85% (E85). Such cars are available from all popular manufacturers and by the end of 2007 four million FFVs were on the road. Brazil was by far the largest producer of ethanol in the world until 2006, when the USA went ahead.

In 1978 the USA established the so-called *Gasohol* program, which introduced E10, a blend of petrol with 10% ethanol. This has the advantage that no separate distribution system is necessary and motor adaptations are minor. Today practically all cars in the US can run on it. Most ethanol plants were set up in the Midwest states where the raw material is grown, which is maize. Ethanol made from maize and other grains has a valuable by-product called DDGS, which contains the germ and husk of the grain, as only the starch is used in the ethanol process. DDGS is utilized as an animal feed instead of e.g. soy beans. The by-products of ethanol production play an important role not only in the economics of the plant but also in the greenhouse gas (GHG) balance, and have considerable side effects in substituting animal feed (imports). Under the impact of legislation with regard to energy independence, clean air and environmental issues the output rose steadily and the US is now the world's largest producer. With 130 plants in operation and around 50 projects announced or under construction the volumes will multiply over the years to come. Currently development is slowing down, however, as high feedstock costs and low ethanol prices put hard pressure on plant viability. There are also transport bottlenecks to resolve. The larger part of the plants are in the maize belt and the rail systems there are used to capacity.

On the way: European Union

In Europe early measures mainly targeted surplus agricultural products. In the 1980s so-called "blind mixing" with up to 5% ethanol was carried out. In France surplus wine was distilled and processed to ETBE for use as a petrol additive. Later Spain and Sweden stepped in. In 2003, under the pressure of environmental issues (Kyoto protocol, "peak oil", climate change) the European Commission adopted the *Biofuel Directive* with indicative targets on the share of renewables. The 2005 target (2%) was only reached in 2007 and strong efforts will be necessary to achieve the 5.75% target for 2010. The latest proposal for a directive by the Commission foresees a binding target of 10% by 2020. It also contains strict sustainability standards, with a minimum 35% GHG reduction and biodiversity criteria for nature protection. The EU biofuel industry nevertheless welcomes the proposal despite its demanding standards and is confident it can comply with it in order to improve the image of biofuels. Europe relies on a variety of different feedstock for ethanol production, including sugar beet, maize, rye and, most commonly, wheat. Around 35 plants provide an annual potential capacity of 3.5 billion litres, but only little more than half of this was used in 2007. There are another 30 plants planned or under construction, but for the same reasons as in the USA, investor interest has declined.

Global bioethanol production and forecast

In 2007 the global bioethanol production approached 50 billion litres, which represents a doubling of the figures for 2002. The production in the USA is estimated at 28 billion litres (2006: 18.5, forecast for 2010: 50.6). Brazil had an estimated annual output of 19 billion litres (2006: 17.3, forecast for 2010: 27.5) and the European Union contributed below 2 billion litres in 2007 (2006: 1.59, forecast for 2010: 7.4). The real figures for 2007 available up to now show a tendency below the preliminary estimates. This is already a consequence of the high raw material costs which hit the biofuel industry in particular, as shown below.

Old benefits and new challenges

A number of benefits and positive impacts have been associated with bioethanol:

- Energy security: less dependence on imported finite oil at constantly increasing prices;
- Air quality: due to cleaner combustion;
- Rural development: utilisation of surplus agricultural products;
- Climate protection: reduction of greenhouse gas emissions from road transport.

Recently, however, a number of concerns have been raised:

- Energy balance? – The production technology has come a long way since the beginning of biofuel programmes in the 1980s. Saving energy is a basic principle in modern plant design, not only to save costs but also because it is closely connected with GHG emission savings. With modern technology bioethanol production has a clearly positive energy balance.
- Sustainability? – Issues like biodiversity, etc. are thoroughly addressed in the EU Directive proposal. The biofuel sector will be the first sector (including food) that needs to produce under very stringent conditions. Biofuels used in the EU will be the most sustainable in the world.
- Costs? – Subsidies and tax breaks are necessary to overcome the market entry barrier. Further investment is needed in this sector to promote research and development. On the other hand, the oil price for one barrel was 10 USD ten years ago; at the time of this presentation it was 110 USD and meanwhile (May 2008) it has risen to 135 USD, with no end in sight whatsoever.
- Feedstock competition? – There is competition for land resources for food, feed, bioenergy and biochemicals. Feedstocks are addressed in more detail in the following section.

Feedstocks

Bioethanol is made from a wide range of field crops. The first important group of raw materials is starch-containing plants, such as grain, mostly maize, and tubers like cassava. The second group in industrial use consists of sugar-containing plants, like sugar beet and sugar cane. A third type of raw material, cellulose converted to carbohydrates, might play a role in the alcohol industry in the future, though to date there has been no industrial implementation based on ligno-cellulosic raw materials.

Analysing the specific cost of bioethanol production, feedstocks represent the largest share of the production cost. Irrespective of the raw material, plant location or capacity, 55–70% of the total costs are related to the raw material. This fact makes the bioethanol industry itself a victim of price increases. (By comparison, for bread the flour cost has a relevance of only 3%!)

Price development of agricultural products

In the course of 2007, wheat and maize prices more than doubled on the stock exchange. The reasons were manifold. For more than a decade US and European governments had been paying farmers to reduce agricultural production. Serious droughts in Australia and poor weather in Europe led to an unexpected reduction in worldwide stocks in 2007. At the same time a rising grain demand was observed in Asia. With yields down and stocks low the prices went up – and speculation further fuelled the price rise.

Meanwhile, all the indications are that there will be a much greater quantity available. The European Union suspended its set-aside scheme, which – theoretically – will bring another 10% land into use. As a consequence of the reform of the EU sugar regime, more arable land is available for other crops. And last but not least, farmers respond to higher prices in their choice of cultivation.

Although the recent price development seems dramatic, a different impression is received by looking at long-term statistics. These show a drastic decline in the real prices of agricultural products for decades, which are only now returning to the level of 30 years ago. For many years farm-gate prices have tended to be below the cost of production.

Cereal end-use

An important detail in judging price developments is the actual share of bioethanol in the end-use of grain. Currently, only 1.6% of the total EU cereal output is used to make fuel ethanol, 1/3 of which enters the feed/food chain as protein concentrated animal feed (DDGS). This protein replaces imported soy meal. (The EU imports 80%, or 40 million tonnes of its vegetable proteins.) By far the biggest share of EU grain production serves to feed cattle (58.1%). The second biggest consumer of cereals is the food industry (22.1%). It is evident that the influence on price formation of this small share used to produce bioethanol is greatly overrated.

The figures in the US are similar: even if around 20% of the maize output in the US is now used for ethanol production, more than half the maize grown there is consumed by animals. (Note: US figures refer to maize, while EU figures refer to all cereals.)

Future developments

A review of agricultural markets by the European Commission concluded that Europe can do both: nutrition and biofuels. The cereal balance sheet in the EU foresees a rise in production, allowing for a growing share for bioethanol and also for exports.

The focus of research and development scenarios concentrates on managing competition for land resources, increasing the yield per hectare and optimizing grain properties for ethanol production, and on scenarios for next generation biofuels. These latter are in the very early stages of development and great efforts are still necessary to make large-scale commercialisation possible.

Future impacts will be mainly determined by global population growth, prosperity and increased demands for energy. While feedstock prices are certainly decisive for bioethanol, the future of the industry is also influenced by a complex economic and political environment. Biofuels will not be the ultimate answer to cope with the challenge of climate change and peak oil, but they have the potential to provide part of the solution.

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LIMITATIONS OF USING DIFFERENTIAL DISPLAY RT-PCR IN THE CHASE FOR SMOKE-RELATED GENES

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Differential Display RT-PCR was developed before the genomic era to serve as a tool in hunting for genes. Nowadays, applications using state-of-the-art techniques to obtain more information about the whole transcriptome or the genome have rapidly overtaken DD-RT-PCR. This paper will discuss a few of the major drawbacks and limitations of using this once highly valued method.

Key words: Differential Display RT-PCR, gene expression, germination, *Lactuca sativa* L. cv. Grand Rapids, lettuce, smoke

Introduction

The characterization of regulated gene expression in eukaryotic cells is essential for studying cell growth and differentiation, as well as for understanding the molecular mechanisms of stresses and environmental impacts. Differential display was developed for such comparative studies by allowing the systematic and non-biased screening of molecular differences at the level of mRNA expression between or within different treatments or tissues (Liang and Pardee, 1992). The essence of the method is to amplify the messenger RNA 3' termini using a pair of anchored oligo-dT primers and a short primer with an arbitrary sequence. The amplified cDNAs, labelled with fluorescent dye, are then run on a denaturing polyacrylamide gel and visualized. The side-by-side comparison of mRNA species from two or more related samples allows the identification of both up- and down-regulated genes of interest. Originally described by Liang and Pardee (1992), Differential Display PCR (DD-RT PCR) is a conceptually attractive technique to examine differential gene expression. Encouraged by the promise of enhanced sensitivity, thousands of investigators have applied this technology. However, few novel genes of interest have been described, indicating that the method has failed to deliver on its promise. Increased utilization has identified its limitations, which include: variable (low) reproducibility (Liang and Pardee, 1992); a significant incidence of false positives (Tiao et al., 1996; Yang et al., 1996); under-representation and redundancy of mRNA signals (Linskens et al., 1995); frequent priming by the G/C rich primer at both ends (Graf et al., 1997; Hadman et al., 1995); a bias towards high copy number mRNAs (Tiao et al., 1996); and a highly labour-intensive procedure. In efforts to unravel the mode of action of smoke on germinating lettuce achenes, Differential Display RT-PCR was used to obtain additional knowledge on smoke-induced genes. Aerosol smoke and smoke-water can break dormancy and promote seed germination of many plant species in fire-prone associations and crops (Adkins and Peters, 2001). To date, little is known about the possible modes of action and the molecular background of the smoke effect (Baxter et al., 1994). A series of experiments, including DD-RT-PCR, microarray, gene and promoter functionalisation tests, were used to elucidate the fundamental characteristics of smoke action. The present paper will discuss the major limitations of DD-RT-PCR.

Materials and methods

Plant material and growth conditions

For RNA isolation, achenes (seeds) of *Lactuca sativa* L. cv. Grand Rapids (150 mg) were germinated in an illuminated or dark environmental chamber (20°C) on tissue paper placed in Petri dishes. One batch of seeds was treated with 3 ml water (control) and the dishes were wrapped in non-transparent aluminium foil (dark). The other batch was treated with 3 ml 1000× diluted smoke extract and then wrapped (dark). The smoke extract was prepared from burnt *Themeda triandra* Forssk. (Poaceae), according to the method outlined in Baxter et al. (1994). Samples were harvested 3, 5, 7, 9, 12 and 24 h after treatment. The germinated achenes were not removed from the Petri dishes. Identical conditions were applied for the germination time course tests, except that 200 seeds were used as starting material.

RNA isolation

Total RNA was isolated from germinating Grand Rapids lettuce seeds (germinated seeds were not removed from samples prior to extraction of RNA) using the Qiagen RNeasy Plant Mini Kit (Qiagen). The RNA was then treated with RNase-free DNase I (Promega) and mRNA was purified using an Oligotex mRNA Mini Kit (Qiagen) according to the manufacturer's instructions. The concentration of RNA was determined with a Nanodrop ND-1000 spectrophotometer (NanoDrop).

Fluorescent differential display RT-PCR (FDD)

FDD was performed as described previously in the RNAimage protocol (GenHunter) with some modifications. First-strand cDNAs were synthesized from each mRNA sample (200 ng) using three different fluorescein-labelled 3'-anchored oligo(dT) primers (5'-fluorescein-GT$_{12}$N-3', n = G, C or A) and the Revertaid First Strand cDNA Synthesis Kit (Fermentas). Dilutions (10×) of cDNAs were amplified by PCR using combinations of fluorescein-labelled anchored and arbitrary primers (HAP 1-20, Genhunter). The conditions for PCR were as follows: 94°C for 3 min, followed by 40 cycles of 94°C for 30 s, 40°C for 2 min and 72°C for 1.5 min, with an additional extension step at 72°C for 5 min. Electrophoresis was carried out on sequencing gel apparatus (Thermo Electron Corporation). Samples were run for 2 h at constant 75 W and detection of the PCR products was performed with a Typhoon Trio+ imager (Amersham Biosciences). In order to facilitate the exact excision of bands of interest, the gel was stained with silver nitrate. The 6% polyacrylamide gel was placed in 10% acetic acid for 20 min with gentle shaking. The gel was washed with water three times, each for 2 min, and stained in a solution containing 0.1% silver nitrate and 0.15% formaldehyde for 30 min. After a quick rinse with water the patterns were revealed by adding the developer (3% sodium carbonate, 0.15% formaldehyde, 0.1% sodium thiosulphate). The reaction was stopped with 10% acetic acid and the gel was washed with water three times and dried at room temperature. Differentially displayed bands were excised and eluted into distilled water, purified and then reamplified by PCR with the appropriate pairs of primers. The products of reamplification were purified with a PCR purification kit (Qiagen) and subcloned into the pGEM-T vector (Promega). For each reamplified fragment, several *E. coli* colonies were chosen, and inserted fragments from these colonies were amplified by PCR. The sizes of inserts were determined by a comparison of mobilities with the isolated band of the original FDD samples. Several independent clones with inserts of the expected size were selected, gel-purified (Qiagen) and sequenced (ABI 3100 Genetic Analyzer).

Real-Time PCR

Lettuce mRNAs (200 ng) were reverse transcribed with the RevertAid first-strand cDNA synthesis kit (Fermentas). Real-time PCR was performed with an Applied Biosystems 7500 real-time PCR system using SYBR Green detection chemistry (Applied Biosystems) and gene-specific primers. The experiment consisted of three independent biological replicates and all reactions were performed in quadruplicate. Specific product amplification was confirmed by T_m analysis using the Dissociation Curve option. PCR efficiency (derived from the log slope of the fluorescence versus cycle number in the exponential phase of each amplification plot) for all primer pairs ranged from 95.5% to 98.0%. Lettuce actin (AY260165) and GAPDH (AF162202) were also selected as potential internal controls and their expression was checked using PCR and microarray data (data not shown). Based on the preliminary findings, actin was selected and used in further experiments. The relative ratio of threshold cycle (Ct) values between the actin and the specific genes and their standard deviations were calculated for each sample. The 3W (3 h water control) samples were used as calibrators.

In silico analysis of the sequences

Sequence alignments were performed with the ClustalW module of the EBI server (http://www.ebi.ac.uk).

Results and discussion

Isolation of smoke-induced and repressed genes

A differential display approach was used to isolate genes from germinating Grand Rapids lettuce achenes whose transcription is affected by smoke treatment. The cDNA fingerprints of smoke-treated germinating lettuce seeds were compared with those of water-treated seeds kept in the dark. The samples were harvested 3, 5, 7, 9, 12 and 24 h after treatment. First-strand cDNA produced from the RNA extracted from the above-mentioned samples was amplified with a combination of arbitrary primers (H-AP 1–16) and 12-nucleotide anchor primers (H-T_{11}-N, N = G, C, or A). A representative differential display pattern is shown in Figure 1.

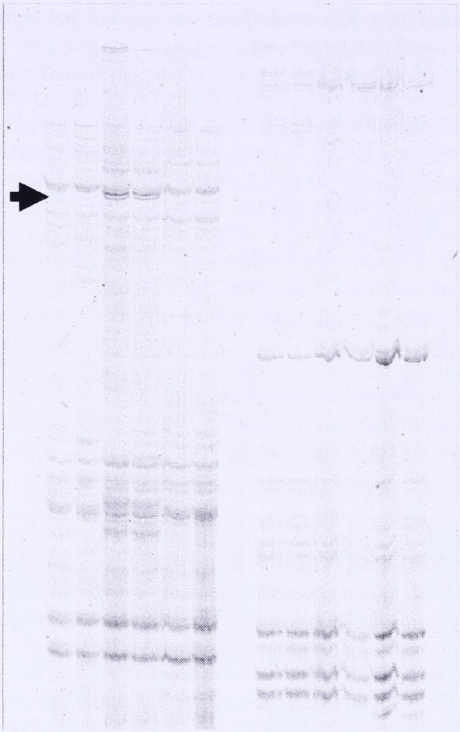

Fig. 1. A typical representation of a DD-RT-PCR gel. The arrow indicates a false positive band

The amplification products of the RNA preparations from one batch of control seeds treated with water for 3, 5, 9, 12 and 24 h are shown in odd lanes and the corresponding samples treated with smoke water are loaded in even lanes. As in Figure 1, 200–250 bands can be seen in every lane, but only a few of them can be regarded as differentially expressed according to the signal intensity. In the smoke-water vs. water-treated controls placed in the dark a total of 27 cDNA fragments with altered patterns were detected, cloned and sequenced. Most bands were present in all twelve lanes, providing an important check on the reaction in general, as major differences between the lanes probably represent a fundamental problem with the reaction. Sixteen out of 27 fragments showed no true altered gene expression pattern after smoke treatment. The false positive bands were only present in one sample and no further occurrence was detected in the repeated experiments, but these would have been classified as a true positive if the experiment had not been performed in triplicate. All the real false positives (8 out of 16) were short (150–200 bp) fragments with no sequence similarity to known genes deposited in databases. The spurious false positives (the other 8 fragments) were identical or highly similar to known sequences and showed a treatment-dependent expression pattern, but not in the triplicates. The importance of this phenomenon is that these genes are not false positives, because they are truly differentially expressed, but not in response to the manipulation under investigation, being affected by other factors. In the present system, about 20% of all the differentially expressed genes were found to be of this type, i.e. they are differentially expressed from one sample preparation to the other (Fig. 1). Real-time PCR confirmed these assumptions, as the transcript abundance of these genes was not consistent in all the triplicates (Fig. 2). The Real-time PCR validation revealed that 11 fragments were true positives, as they showed a parallel expression pattern with the DD-gel. Among these clones, selected for further study (discussed in other publications), ten showed significant homology to known genes or gene families, while the other showed similarity to an unknown *Arabidopsis* gene. Performing all experiments in triplicate, using different samples from independent experiments, has the advantage of reducing the incidence of false positives by allowing the identification of genes that are consistently regulated by the manipulation under study. In addition, it avoids the isolation of genes that are truly differentially regulated but whose regulation is not related to the manipulation under study but to other factors specific to the individuals under study. This approach allows the reliable identification of genes that are differentially expressed in a quantitative fashion without being completely absent in any lane. Such quantitatively regulated genes can be difficult to identify using the conventional single preparation approach, because a quantitative difference in band strength is often difficult to distinguish from loading differences between the lanes, even when the strength of non-regulated bands is taken into account.

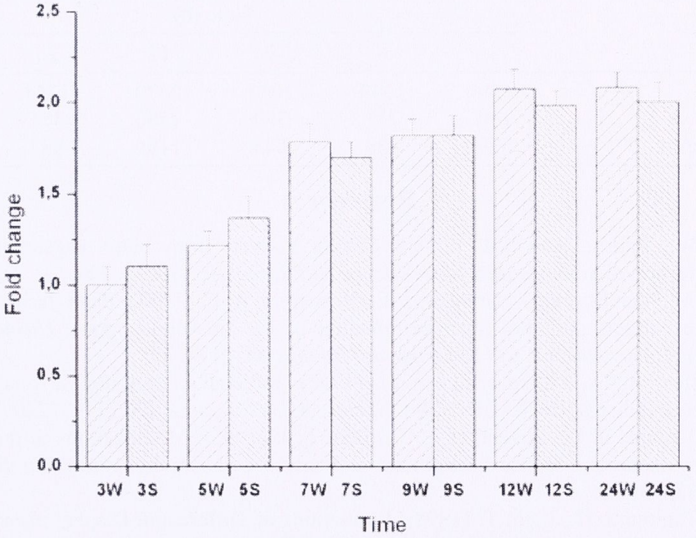

Fig. 2. Expression analysis of the fructose-1,6-bisphosphatase gene in response to smoke treatment. Relative transcript abundance was calculated and normalized with respect to the actin transcript level. The 3 h control was set as calibrator. Data shown represent mean values obtained from four independent amplification reactions ($n = 4$). The experiment was repeated three times with similar results. On the plots, W indicates water control (kept in dark), S indicates smoke-treated samples (kept in dark)

As can be seen, only 11 fragments regarded as true positives were chosen in connection with smoke action. It is anticipated that far more genes could be expected to be smoke-related. Because of its limitations the DD approach only allowed the most pronounced genes involved in smoke action to be separated, and transcripts with lower abundance remained hidden, making it impossible to plot possible regulatory pathways. Thus, the high occurrence of false positives and the evidence that aberrant priming at both the 5′ and 3′ ends results in competition in the PCR, precluding the detection of messages, even those which are abundantly expressed (Ledakis et al., 1998), further narrows the number of possible candidates. Not only the significant incidence of false positives but also the under-representation and redundancy of mRNA signals are further problems with the DD-RT-PCR approach. A microarray experiment was conducted on germinating maize kernels to obtain the high density transcriptome of smoke-treated kernels (discussed elsewhere). The experiment resulted in far more possible candidate genes even after filtering the germination-related transcripts (Table 1). While DD may be successfully applied in some settings, the accumulating evidence indicates that even after extensive screening is performed, only the tip of the iceberg has been explored.

Table 1

Up- and down-regulated genes in the maize transcriptome induced by smoke extract

	Time (h)					
	3	6	9	12	24	27
Total	659	1549	1012	2479	1253	3321
Up-regulated	130	711	369	1280	371	1623
Down- regulated	529	838	643	1199	882	1698

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GENETIC MODIFICATION OF CEREALS IN THE AGRICULTURAL RESEARCH INSTITUTE OF THE HUNGARIAN ACADEMY OF SCIENCES

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Research with transgenic plants in the Agricultural Research Institute of the Hungarian Academy of Sciences is primarily related to applications that are essential for the genetic improvement of cereals. The two main directions are connected to wheat and maize breeding and are focused on improving agronomic and nutritional traits. This paper highlights experiments in these areas, which are conducted in national as well as international collaborations. The transparency of this work is ensured by the dissemination of information about approved confined field tests to the public via the internet.

Introduction

One of the basic scientific tasks of the Agricultural Research Institute of the Hungarian Academy of Sciences is to investigate the characteristics of wheat and maize and to reveal their biochemical and genetic backgrounds. The knowledge acquired is then exploited in cereal breeding. In addition to conventional breeding methods, an increasing role has been played over the last ten years by modern biotechnological procedures (e.g. genetic modification or gene transformation) which, among other things, allow the effects of individual genes to be analysed. The development of genetically modified plants is dependent on the availability of reliable gene transfer techniques adapted to local laboratory conditions. This purpose is served by experiments involving reporter genes (e.g. *gusA* and *gfp*) or the *bar* selectable marker gene, where the success of gene transfer is indicated by a simple colour reaction or by the presence of herbicide resistance. "Useful" genes can only be transferred after the optimisation of the methodology, which must be carried out individually for each plant species intended for genetic modification (in the present case wheat, barley and maize). In Martonvásár the aim of genetic modification is partly to improve the agronomic traits and environmental resistance of these plants, and partly to modify breadmaking quality or nutritional value to satisfy the requirements of the processing industry.

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Research on wheat

Among the agronomic traits of wheat, the main focus is on resistance to powdery mildew and frost tolerance. Within the framework of an EU project, in cooperation with the University of Zurich, work is underway on the introduction of a gene responsible for powdery mildew resistance into winter and spring wheat varieties, followed by the monitoring of its incorporation into the wheat genome and its ability to exert a positive effect and make the test plants resistant to powdery mildew.

The cold stress-related gene regulatory pathway is one of the most thoroughly investigated regulatory systems in the plant kingdom. It has been proved that the *CBF* genes, coding for transcription factors, are among the key regulators for low temperature stress response. Recently it has demonstrated that four *CBF* genes are the main regulators in wheat (Vágújfalvi et al., 2005). To directly prove the involvement of these genes in frost tolerance, *Arabidopsis*, rice, wheat and barley plants were considered for transformation.

Due to methodological advantages, the model plant *Arabidopsis* was the first to be transformed with the candidate *CBF* genes (Fig. 1) in collaboration with Corvinus University, Budapest, Hungary. The plants were transformed with three candidate genes using the floral dip method. The T2 generation was subjected to frost tests and several transgenic lines with increased frost tolerance were identified. Plants with increased tolerance will be verified for transgene expression and copy number. Cereals have been transformed in cooperation with the Agricultural Biotechnology Center, Gödöllő, Hungary and the John Innes Centre, Norwich, UK, using the biolistic method in the case of wheat and rice. In these experiments the candidate genes were driven by the constitutive maize ubiquitin promoter, but tests on the effectiveness of a cold-inducible wheat promoter, *WCS120*, are also planned. Currently the transformants are being tested for the presence of the transgenes. Since wheat transformation is time-consuming and labour-intensive, barley plants were transformed using the *Agrobacterium*-mediated method. The verification of successful transformation is now in progress. The participation of the candidate *CBF* genes in the control of frost tolerance is also being screened with the RNAi technique.

In addition to agronomic traits, the breadmaking quality of wheat is also of major importance. This depends chiefly on the composition of the storage proteins (e.g. HMW glutenins and gliadins). It has long been known that better quality products can be prepared if certain glutenin subunits are present in higher quantities. To confirm this observation, spring wheat varieties were transformed with glutenin subunits 1Dx5 and 1Ax1 at Rothamsted Research, UK, after which the breadmaking quality of the transgenic plants was examined in Martonvásár (Rakszegi et al., 2005). As the result of genetic modification the quantity of 1Dx5 glutenin subunit in the flour was found to increase fourfold, leading to extremely strong dough (Fig. 2). This flour can be used in practice by mixing it with other types of flour. The 1Ax1 transformation led to more stable dough which softened at a slower rate. The incorporated wheat genes and the encoded traits proved to be inherited in a stable manner from one generation to the next.

In order to improve the nutritional value of wheat, transformation was carried out (in cooperation with the Plant Physiology Department of Eötvös Loránd University, Budapest) with the *AmA1* gene coding for a seed albumin (Raina and Datta, 1992) containing a high proportion of essential amino acids, which humans are unable to synthesise and which must thus be consumed as food. The gene used for the genetic modification originated from *Amaranthus hypochondriacus*, commonly referred to as the cereal source for people allergic to wheat flour and other foodstuffs. The gene transfer resulted in a substantial rise in the essential amino acid content of the flour, with increases of around 6% for lysine, 2.8% for threonine and 3.8% for tyrosine, thus improving the nutritional value of the wheat.

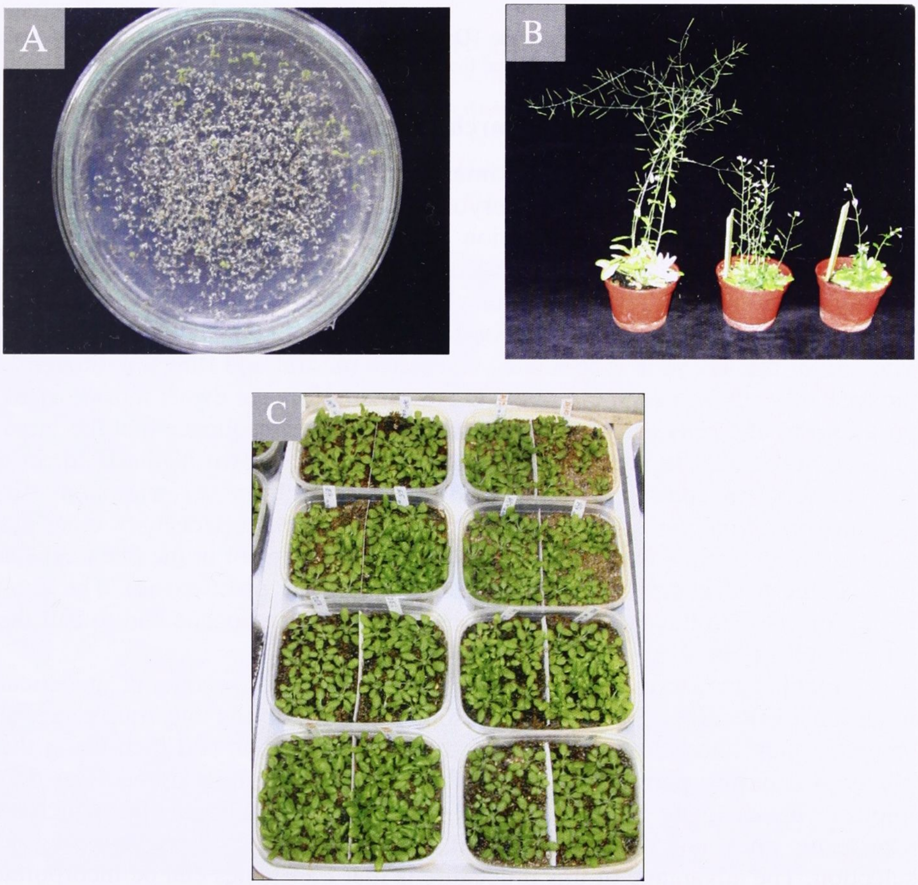

Fig. 1. Arabidopsis transformation. A: selection for *CBF* transformants, B: T1 generation, C: Propagation of the T2 generation

Fig. 2. Effect of 4-fold over-production of the 1Dx5 storage protein subunit on breadmaking quality: bread baked using the control flour (left) and that baked using flour from GM plants (right)

Research on maize

In gene transformation experiments on maize, genes are transferred into calli induced from immature embryos, using a gene gun, or into calli of microspore origin, via cocultivation with *Agrobacterium tumefaciens*. As microspores are haploid, the transgenes in plants regenerated from calli of microspore origin can be made homozygous and non-segregating within a single generation, due to the spontaneous or artificial doubling of the genetic material. The aim of this research is to induce resistance to viral and fungal pathogens. In the case of viruses, a segment of the coat protein of maize dwarf mosaic virus is produced by the cells of transgenic plants, with the consequence that the virus is unlikely to be able to reproduce when the plants are grown in the field. In the case of fungi, a chitin-decomposing enzyme (endochitinase) originating from *Trichoderma hamatum* (Fekete et al., 1996), a fungus that parasitises other fungi and is used in many countries in organic farming, is present in the plant cells and is thus able to decompose fungal hyphae invading the plant tissues. The aim of both projects is to develop maize hybrids resistant to pathogens and to suit them for use in the field.

Field gene transfer is another possibility for producing genetically modified plants. This technique is based on incorporating the transgenes into "model" plant lines ideally suited for gene transformation and then using these plants as crossing partners in the field transformation programme. The donor line carrying the gene is single-crossed with the recipient lines, after which new transgenic lines are developed through several cycles of backcrossing and selection. The advantage of this procedure is that transgenes can be incorporated into maize lines which have commercial value but are not suited to current gene transformation techniques, preventing them from being used for genetic modification up till now.

In Martonvásár the field breeding programme is aimed at the transfer of genes responsible for resistance to western corn rootworm (*Diabrotica virgifera virgifera*) and herbicides. In cooperation with Monsanto Hungária Co. Ltd., the gene coding for the Cry3Bb1 delta-endotoxin protein of the bacterium *Bacillus thuringiensis* subsp. *kumamotoensis* (Donovan et al., 1992) is transformed into Martonvásár maize lines, allowing a specific protein to be produced in the plant cells which destroys both the larvae and imagos of corn rootworm when they attack the plant tissues (Fig. 3). The results of recent biosafety research have shown that this protein exerts no negative effect on non-target organisms in the field (Rauschen et al., 2008). A modified maize gene (*epsps*) is responsible for the herbicide resistance in this programme. This provides protection against glyphosate, the active ingredient in Roundup, a total herbicide capable of destroying practically every weed on the growing area, with the exception of the genetically modified maize.

The field release of the plants arising from these research projects is for experimental purposes, aimed at investigating the expression and inheritance of the incorporated genes. The research institute has a licence to carry out such confined field experiments, details of which are publicly available in the database of authorised GMO releases in Hungary, to be found on the website of the Agricultural Biotechnology Center in Gödöllő (http://biosafety.abc.hu).

Fig. 3. Comparison of the damage caused by western corn rootworm: the resistant GM line is symptom-free (left), unlike the susceptible control (right)

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EFFECTS OF FORE-CROP FERTILIZATION ON THE YIELD AND QUALITY OF KIDNEY BEANS UNDER VEGETABLE CROP ROTATION CONDITIONS

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Kidney beans (*Phaseolus vulgaris* (L.) Savi.) ssp. Nanus cv. Lodi were grown as a mid-early second crop after lettuce on an alluvial meadow soil with pH($_{H2O}$) = 6.4 and a plant density of 22 plants per m^2. The bean crop was grown without fertilization, but the lettuce crop was treated with mineral, organic or foliar fertilization. The application of different fertilizer sources to the fore-crop resulted in high yields of the subsequent bean crop, with good quality parameters and without polluting the arable soil layer with fertilizer residues. Foliar fertilization applied to the fore-crop resulted in a lower accumulation of nitrates in fresh beans in comparison with the other fertilizer sources. Organic fertilization supplied during the cultivation of the previous crop had a stronger effect than mineral fertilization on certain quality parameters of the kidney bean plants, such as dry matter, vitamin C content, and the accumulation of nitrates.

Key words: kidney beans, lettuce, fertilizer sources, crop rotation, quality parameters

Introduction

Several factors are responsible for the quality of fresh vegetable produce. Among these factors, fertilizer forms and rates play an important role in ensuring an optimal nutritional regime for successful plant development and in influencing the yield and quality of vegetables (Sidiras et al., 1999). Horticulture may result in the accumulation of nitrogen fertilizer residues in the root zone after the crop harvest. Eddy (2000) and Stancheva et al. (2004) reported on the effects of foliar fertilization on the duration of the plant growth period and on improvements in fruit quantity and quality. Reducing the supply of fertilizers and crop cultivation after a fore-crop is also a promising way to improve the quality of vegetables. When lettuce was grown as a third crop in the rotation, the maximum inclusion of nitrogen in the nutrient cycle was observed and the nitrogen content in the soil was restored to close to its background values (Stoicheva et al., 2002).

The objective of the study was thus to estimate the after-effects of applying different fertilizer forms (with equivalent nitrogen content) to the fore-crop on the yield and quality of the next crop of kidney beans and on the amount of fertilizer residues in the arable soil layer.

Materials and methods

Kidney bean plants (*Phaseolus vulgaris* (L.) Savi.) ssp. Nanus cv. Lodi were grown as a mid-early second crop after lettuce between 10 May and 28 July 2005. The plants were grown in the experimental field of the N. Poushkarov Institute in Tzalapitca (Plovdiv region) on an alluvial meadow soil with the following agrochemical characteristics: $pH(_{H2O})$: 6.4, soluble nitrogen: 12.09 mg kg^{-1} soil (5.85 mg kg^{-1} NH_4^+-N and 6.24 mg kg^{-1} NO_3^--N), P_2O_5: 73 mg kg^{-1} soil, K_2O: 179 mg kg^{-1} and organic matter: 0.70%. All the treatments were arranged in a randomized complete block design with four replicates. The experimental plot area was 40 m^2, consisting of 12 rows with 75 plants in each row, giving a plant density of about 22 plants per m^2. The kidney beans were grown without fertilization after a lettuce crop fertilized with the following fertilizer forms and amounts: B_1 – control without fertilization; B_2 – mineral fertilization with 150 kg ha^{-1} N, 100 kg ha^{-1} P, 100 kg ha^{-1} K ($N_{150}P_{100}K_{100}$); B_3 – organic fertilization with farmyard manure (24 t ha^{-1}); B_4 – foliar fertilization with Agroleaf ($N_{20\%}P_{20\%}K_{20\%}$). All the fertilizer forms supplied contained equivalent nitrogen amounts. In the variants with mineral fertilization, nitrogen fertilizer was applied as ammonium nitrate, 2/3 before lettuce planting and 1/3 as top dressing. Phosphorus was applied as triple superphosphate before planting and potassium as K_2SO_4, half before planting and half as top dressing. The composition of the decomposed sheep manure was: 0.64% total N, 1.84% total P_2O_5 and 0.51% total K_2O. Agroleaf® (Scotts Company, Ohio, USA, distributed by VLADI Co. in Bulgaria) containing a 20:20:20 ratio of N:P:K + all important microelements in chelated form (0.1% Fe, 0.06% Mn, 0.06% Cu, 0.06% Zn, 0.02% B), was applied three times during the vegetative growth stage at 10-day intervals, starting 15 days after planting. Agroleaf® was applied by spraying under high pressure at rates of 5 kg ha^{-1} or 0.5% solution. When the kidney beans were harvested the following parameters were measured: fresh biomass yield, dry weight, cellulose after Kyurshner (Ermakov et al., 1952) and total soluble sugars (Dubois et al., 1956). Vitamin C content was determined using an HPLC system equipped with a Supelco C-18-DB column (150 mm, 4.6 mm, 3.0 µm) and a UV/VIS detector 245, with an eluent flow rate of 1 ml min^{-1}, a sample volume of 10 µl and a solvent composition of KH_2PO_4 (pH=3.0). Nitrachek (Hawk Creek Laboratory Inc., USA) was used to determine the content of nitrates, and a leaf tissue nitrate quick test was made. The kidney bean yields were calculated from 60 plants per plot. Nitrate reductase (NR, EC 1.6.6.1) was measured *in vivo* according to Klepper et al. (1971). When the crop was harvested, soil samples were collected for the determination of soil mineral nitrogen (spectrophotometrically after Kjeldahl digestion) and of phosphorus and potassium using the acetate-lactate method (Ivanov, 1984).

Data are expressed as means ± standard error, where n=4. Comparison of means was made by the Fisher LSD test ($P \leq 0.05$) after performing multifactor ANOVA analysis. The Statgraphics software (statistical package version 5.0) was used for statistical analysis.

Results and discussion

The effect of applying various fertilizer forms to the previous crop on the yield of kidney beans grown without fertilization is shown in Figure 1. The results of dispersion analysis did not show any significant differences between the tested variants of fertilization to the lettuce fore-crop, proving the balanced role of kidney beans as a subsequent crop. Despite the lack of fertilization, the yields obtained were fairly high. According to some authors (Peev, 1985; Shaban, 2002) average kidney bean yields from low-growing varieties varied from 8 to 20 t ha^{-1}, depending on the variety and way of cultivation. Therefore, the yields obtained in the present experiment were higher than the average values.

Some studies (Peev, 1985; Tonev et al., 1999) on beans and other legume crops showed that quality parameters and especially dry matter content were genetically determined and that several environmental factors had a weak impact on these parameters. Dry matter content did not significantly differ between the variant in which foliar fertilization was applied to the fore-crop and the control variant (Table 1), where the lowest value was obtained. The highest values were observed in the variant given organic fertilizer, followed by the variant given mineral fertilization to the fore-crop. Hence, organic and mineral fertilizers supplied to the previous lettuce crop positively affected the dry matter content of bean pods. The vitamin C content in the bean pods varied between 105 and 130 mg kg^{-1} fresh weight and significantly higher values were observed after organic fertilization. The vitamin C content in all the variants was high, completely satisfying the daily requirements of children, based on reports that 100 g boiled garden beans contain 5 mg vitamin C. The content of soluble sugars in the bean pods varied between 9.6 and 11.1 mg kg^{-1} fresh weight. A significantly lower value was measured after mineral fertilization, where the highest cellulose content was observed (Table 1). Gonnella et al. (2000) reported that inappropriate fertilization led to quality deterioration, with high levels of crude protein, cellulose and nitrates and a reduction in carbohydrates and vitamins.

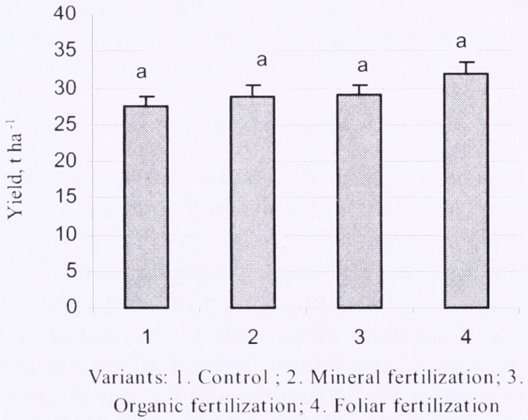


Fig. 1. Yield of kidney beans (t ha^{-1}) grown as a subsequent crop after lettuce given various fertilization treatments. Different letters indicate significant differences assessed by the Fisher LSD test ($P \leq 0.05$) after performing ANOVA multifactor analysis

Table 1

Quality parameters of kidney beans

Variants	Dry weight (%)	Vitamin C (mg kg^{-1} FW)	Soluble sugars (mg kg^{-1} DW)	Cellulose (% dry matter)	NO_3^- content (mg kg^{-1} FW)
B_1 control	$7.14 \pm 0.35^{*a}$	109 ± 5.48^{a}	10.4 ± 0.51^{ab}	11.26	116 ± 5.8^{a}
B_2 mineral fertilization	8.33 ± 0.40^{b}	112 ± 5.60^{a}	9.6 ± 0.48^{a}	11.88	202 ± 10.1^{b}
B_3 organic fertilization	9.66 ± 0.47^{c}	130 ± 5.63^{b}	10.8 ± 0.53^{b}	10.58	266 ± 13.3^{c}
B_4 foliar fertilization	7.33 ± 0.36^{a}	105 ± 5.24^{a}	11.1 ± 0.55^{b}	10.07	190 ± 9.5^{b}

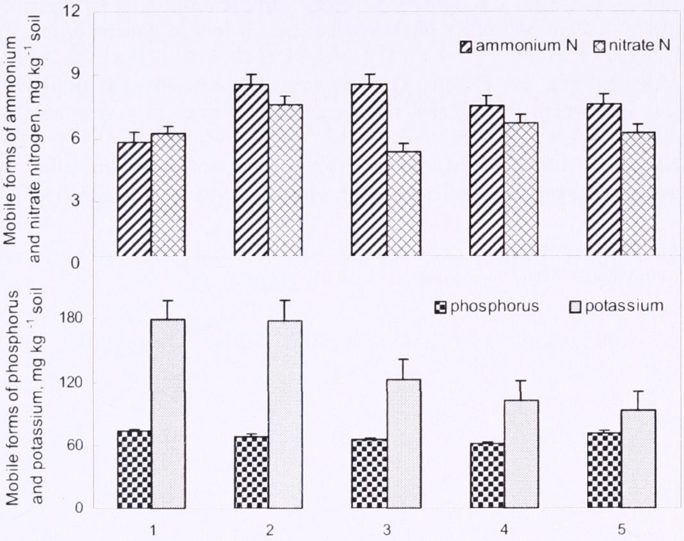
FW: fresh weight; DW: dry weight; *Values are means ± S.E., n=4; Different letters indicate significant differences assessed by the Fisher LSD test ($P \leq 0.05$) after performing ANOVA multifactor analysis

The nitrate content of the bean pods in the control and after foliar fertilization to the previous crop remained below the Bulgarian limit of 200 mg kg^{-1} fresh weight. The accumulation of nitrates above this level was observed after mineral and organic fertilization (202 and 226 mg kg^{-1}, respectively). The high nitrate concentration after organic fertilization could be due to the slow release of nitrogen during the mineralization of organic nitrogen in the farmyard manure supplied to the previous crop of winter lettuce, grown under conditions with low soil and air temperatures.

The mobile forms of the main macronutrients, ammonium and nitrate N, P and K, indicating the amounts of fertilizer residues in the arable soil layer after the harvest of the fore-crop (lettuce) and the kidney beans, are shown in Figure 2. An increase in mobile nitrogen was observed in all the experimental variants in comparison with the initial level, especially in the control plants. This could be due to the additional nitrogen-fixing activity of the nodules of bean plants. Differences in the P levels among the variants and compared to the initial levels were negligible (Fig. 2). Residual K amounts after the kidney bean harvest were lower than the initial levels. The lack of increase in macronutrient residues after the second crop harvest indicated the adequate choice of crop and nitrogen rates supplied to the fore-crop.

Conclusions

It could be concluded from the results that the application of different fertilizer sources and rates to the fore-crop produced high yields of the subsequent kidney bean crop, with good quality parameters and without polluting the soil with fertilizer residues. The application of organic fertilization to the previous crop had a stronger effect than mineral fertilization on a number of quality parameters of the next crop, such as dry matter, vitamin C content, soluble sugars and nitrate accumulation. Foliar fertilization applied to the fore-crop did not have a positive influence on the yield or on other quality parameters, but resulted in the lower accumulation of nitrates in fresh bean pods in comparison with the other fertilizer sources. To avoid the accumulation of nitrates in the bean pods above the authorized limit, it is recommended that no more than 24 t ha^{-1} organic fertilization should be applied to the fore-crop, especially under conditions of low soil and air temperatures.



Variants: 1. Initial soil level, 2. After control treatment, 3. After mineral fertilization, 4. After organic fertilization, 5. After foliar fertilization.

Fig. 2. Changes in ammonium and nitrate mineral nitrogen and in mobile P and K forms in the arable soil layer after harvesting kidney beans. Bars indicate significant differences assessed by the Fisher LSD test ($P \leq 0.05$) after performing ANOVA multifactor analysis

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GENE EFFECTS AND MEAN PERFORMANCE OF NITROGEN AND PHOSPHORUS USE IN WHEAT AFTER INOCULATION WITH ARBUSCULAR MYCORRHIZA FUNGI AND *Azotobacter chroococcum* UNDER LOW INPUT CONDITIONS

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The present investigation was conducted to study the impact of bio-inoculants under low input field conditions on the magnitude and direction of gene effects and the mean performance of nitrogen (N) and phosphorus (P) use in wheat. Three wheat cultivars suitable for different agro-ecological conditions, i.e. WH 147 (low mineral input), WH 533 (water deficit), Raj 3077 (high mineral input), and six generations (P_1, P_2, F_1, F_2, BC_1 and BC_2) of three crosses, namely WH 147 × WH 533, WH 533 × Raj 3077 and WH 147 × Raj 3077, were evaluated in a randomized block design with three replications under low input field conditions (80 kg N + 40 kg P + 18 kg $ZnSO_4$ doses applied in each treatment) with three treatments, i.e. control, inoculation with arbuscular mycorrhiza fungi (AMF, *Glomus fasciculatum*) and dual inoculation with AMF and *Azotobacter chroococcum* (*Azc*). Bio-inoculation with AMF and AMF+*Azc* had a positive impact on the mean performance of all the wheat crosses. The mean performance of AMF was maximum in the cross WH 147 × WH 533 for N and P response (%), N and P use index (%) and P content (ppm), whereas for N and P uptake it was maximum in the cross WH 147 × Raj 3077. The response and use index for N and P were better in the combined AMF+*Azc* treatment in all three crosses. The adequacy of the additive-dominance model for the phosphorus uptake (mg/plant) by all three crosses in all three treatments (i.e. control, AMF, AMF+*Azc*) suggested that additive (d) and dominance (h) gene effects mainly governed the inheritance of this trait. In all cases, digenic interactions were present, where the duplicate type of epistasis prevailed except for the P content in the control in the cross WH 147 × WH 533, where the complementary type of interaction was present. Pedigree selection in crosses WH 147 × WH 533 and WH 147 × Raj 3077 could be effective for breeding pure lines of wheat for sustainable agriculture (low input genotypes responsive to biofertilizers such as AMF and *Azotobacter*).

Key words: wheat, *Azotobacter chroococcum*, arbuscular mycorrhiza fungi, gene effects, nitrogen, phosphorus, low input

Introduction

Microorganisms such as arbuscular mycorrhiza fungi (AMF) and rhizobacteria *Azotobacter chroococcum* (*Azc*) frequently have a stimulative effect on plant growth. Inoculation with AMF has been found to increase the availability of phosphorus and other nutrients in crop plants as it forms a symbiotic association with plant roots, colonizes cortical tissues and extends hyphae into the rhizosphere (Hetrick et al., 1996). Inoculation with *Azc* also complements the wheat-AMF interaction due to its nitrogen fixation, phytohormone production and phosphate-solubilizing properties (Kumar et al., 2001). Root-infecting fungi, such as mycorrhiza, and rhizospheric bacteria, such as *Azotobacter*, especially interact with wheat genotypes to fix phosphorus and nitrogen, respectively. The increased growth of mycorrhizal plants is favoured in soils with low to moderate fertility (Paradi et al., 2003). Greater soil exploration by mycorrhizal roots as a means of increasing P, Zn, Cu, S, etc. uptake is well known. The effects of vesicular-arbuscular mycorrhiza result from their ability to increase phosphorus uptake, but the benefits derived from the infection vary between plant species (Gerdemann, 1975). Although genotypic differences in the response of Indian wheat to AMF and *Azc* inoculation individually or in combination have been reported (Manske et al., 2000; Behl et al., 2003), information on whether such differences are heritable as reflected in cross combinations for nutrient use is scanty.

The present study deals with the effect of AMF and AMF+*Azc* treatments on the nitrogen and phosphorus content and on their uptake, response and use index in three wheat crosses.

Materials and methods

Three genetically diverse wheat genotypes suitable for different agro-ecological conditions, namely WH 147 (low mineral input), WH 533 (water deficit) and Raj 3077 (high mineral input), were involved in three crosses, WH 147 × WH 533, WH 533 × Raj 3077 and WH 147 × Raj 3077, six generations (P_1, P_2, F_1, F_2, BC_1 and BC_2) of which were evaluated in a randomized block design with three replications under low input field conditions with three treatments, i.e. control, inoculation with arbuscular mycorrhiza fungi (AMF) and dual inoculation with AMF and *Azotobacter* (*Azc*). Two rows each of F_1s and parents, ten rows of F_2s and four rows of backcrosses were planted, with row-to-row and plant-to-plant spacings of 30 and 10 cm, respectively.

For AMF inoculation, pearl millet roots infected with *Glomus fasciculatum* were chopped into small pieces and mixed with the soil in the furrows at the time of sowing. *Azotobacter chroococcum* mutant Mac 27 was grown on nitrogen-free Jensen medium (Jensen, 1951) containing 2% sucrose at 30°C for 72 h. For *Azc* inoculation the wheat seeds were first treated with traditional jaggery or molasses solution prior to treatment with charcoal-based *Azc* Mac 27 in a beaker and shaken thoroughly to facilitate the uniform coating of the seeds with inoculum, using a concentration of 10^9 colony forming units (CFU)/ml, determined by the plate count method (Narula et al., 2000). *Azotobacter*-treated seeds were kept in the shade for about one hour for drying before sowing, so that the *Azc* inoculum could adhere to the seeds. For dual inoculation wheat seeds pre-inoculated with *Azc* were co-inoculated with AMF. In both the bio-inoculant-treated plots, the mineral fertilizer dose was the same as in the control (80 kg N + 40 kg P + 18 kg $ZnSO_4$). The soil was a sandy loam, alluvial in nature, having pH 8.5, organic carbon 0.35%, total nitrogen 0.034% and available phosphate 4.2 mg/kg.

Observations were made on five plants of the non-segregating generations (parents and F_1s), fifty plants in F_2 and twenty-five plants in each of the backcross generations of each of the three crosses in each replication to record root length density and AMF infection in the roots. The means and variances of individual families were calculated and used for the estimation of gene effects (Jinks and Jones, 1958). The adequacy of the additive-dominance model was determined by the joint scaling test suggested by Cavalli (1952).

The nitrogen (N) content (ppm) was estimated in the straw and phosphorus (P) content (ppm) in the shoot using an Autoanalyser (Pulse Instrumentation Ltd., Saskatoon, Canada). The N and P uptake (mg/plant) was calculated by multiplying the N and P concentrations in the shoot by the dry weight. The N and P responses (%) were estimated using the following formula:

$$\frac{\text{N and P uptake under treated conditions} - \text{uptake under control conditions}}{\text{N and P uptake under control conditions}} \times 100$$

The N and P use index (%) was estimated with the following formula:

$$\frac{\text{N and P uptake under treated conditions} - \text{uptake under control conditions}}{\text{N and P uptake under treated conditions}} \times 100$$

Results and discussion

Mean performance

In general, bio-inoculation with AMF and AMF+*Azc* had a positive impact on the mean performance of all the wheat crosses. The magnitude of the impact for various traits in different crosses was evaluated by comparing estimated values of the mean 'm' between the control and inoculated treatments of AMF and AMF+*Azc*. For brevity, only crosses which exhibited significant differences for various traits are presented in Table 1. The mean performance of AMF inoculation was maximum in the cross WH 147 × WH 533, followed by WH 533 × Raj 3077 and WH 147 × Raj 3077 for N and P response (%), N and P use index (%) and P content (ppm), whereas for N and P uptake it was maximum in the cross WH 147 × Raj 3077. Inoculation with *A. chroococcum* resulted in an increase in P uptake over the control, which may be due to the bacterial solubilization of insoluble phosphates in soil having high pH (8.5) (Narula et al., 2000). The mean performance of AMF+*Azc* inoculation was the highest in the cross WH 533 × Raj 3077, followed by WH 147 × WH 533, and the lowest in WH 147 × Raj 3077 for N and P use index (%) and for P content and uptake. Similar results were also reported by Singh et al. (2004). For N content and uptake and P response the mean performance of AMF+*Azc* was maximum in the cross WH 147 × WH 533. The N and P response and use index were better when the combined AMF+*Azc* treatment was given in all three crosses.

Gene effects

Non-significant chi-square values in the joint scaling test (Cavalli, 1952) indicated the adequacy of the additive-dominance model for the inheritance of phosphorus uptake (mg/plant) in all three crosses under all three treatments (i.e.

control, AMF, AMF+*Azc*). This suggested that mainly additive (d) and dominance (h) gene effects governed the inheritance of this trait (Table 2). Both additive (d) and dominance (h) gene effects were significant in the cross WH 533 × Raj 3077 for the P content in the control and AMF treatments, whereas for P uptake only dominance gene effects (h) were significant in the cross WH 533 × Raj 3077 in all three treatments and additive (d) effects in the WH 147 × WH 533 cross for the AMF+*Azc* treatment.

Significant chi-square values in the joint scaling tests indicated the presence of digenic interactions in the control for N content and uptake in all three crosses, while for P content these were only present in two crosses, i.e. WH 147 × WH 533 and WH 147 × Raj 3077. Digenic interactions were only prevalent in the control and the AMF+*Azc* treatments for N content, N uptake and P content for two crosses. For the AMF treatment all three crosses exhibited digenic interaction for N uptake, but for N and P content these were present only in two crosses. The estimates of d, h, i, j and l were significant except for N response in the AMF+*Azc* treatments and N use index in the AMF treatment. In all cases digenic interactions were present where the duplicate type of epistasis (D) was present except for P content in the control for the cross WH 147 × WH 533, where the complementary type of interaction (I) was observed (Table 3).

Table 1

Mean performance (m) of six generations of three wheat crosses exhibiting significant differences between control and bio-inoculated treatments for nitrogen (N) and phosphorus (P) macronutrients

Characters	Treatments	WH 147 × WH 533	WH 533 × Raj 3077	WH 147 × Raj 3077	CD (5%)
N content (ppm)	Control	5737.83±20.13	5218.88±18.93*	5210.75±14.88*	
	AMF	5999.84±11.83*	4674.63±16.86	5010.85±12.96*	244.86
	AMF+*Azc*	5560.55±18.05	5249.88±17.93*	4707.21±13.35	
N uptake (mg/plant)	Control	194.78±1.58	189.86±1.44	220.11±1.74	
	AMF	235.45±1.72*	226.43±1.46*	237.02±1.58*	5.27
	AMF+*Azc*	248.11±1.73*	240.03±1.54*	257.71±1.98*	
N response (%)	Control	–	–	–	
	AMF	21.28±1.68	21.00±2.14	15.37±2.11	0.26
	AMF+*Azc*	28.20±2.99*	27.49±2.84*	21.38±2.69*	
N use index (%)	Control	–	–	–	
	AMF	17.66±1.71	17.47±2.43	13.66±1.93	0.24
	AMF+*Azc*	22.54±1.99*	23.17±2.41*	18.83±2.02*	
P content (ppm)	Control	360.37±4.88	365.89±4.72	329.73±4.32	
	AMF	389.45±5.47	–	346.05±4.59	107.16
	AMF+*Azc*	374.77±5.74	391.03±5.23	346.12±4.44	
P uptake (mg/plant)	Control	12.64±0.79	13.84±0.65	14.14±0.89	
	AMF	15.17±0.92	15.17±0.90	–	3.24
	AMF+*Azc*	16.50±0.78*	16.56±0.67	16.32±0.98	
P response (%)	Control	–	–	–	
	AMF	20.18±2.80	18.94±2.05	17.38±1.83	0.35
	AMF+*Azc*	24.62±3.21*	24.50±2.85*	22.01±2.32*	
P use index (%)	Control	–	–	–	
	AMF	18.28±2.42	15.98±2.27	15.26±2.24	0.86
	AMF+*Azc*	20.68±2.54*	21.26±2.74*	18.43±1.47*	

* = Significant at the 5% level

Table 2

Gene effects (additive and dominance) in three wheat crosses for nitrogen (N) and phosphorus (P) in different bio-inoculation treatments

Characters	Treatments	WH 147 × WH 533		WH 533 × Raj 3077		WH 147 × Raj 3077	
		d	h	d	h	d	h
N content	AMF	digenic	digenic	digenic	digenic	190.41±12.71	–358.62±26.71
N response (%)	AMF	digenic	digenic	digenic	digenic	–0.50±2.91	7.38±4.80
	AMF+*Azc*	digenic	digenic	–0.28±2.81	–2.88±4.56	–2.08±2.65	7.22±4.95
N use index (%)	AMF	digenic	digenic	2.19±2.19	1.86±4.56	–0.70±1.81	2.37±4.52
	AMF+*Azc*	–6.29±1.98**	2.29±3.43	–1.02±2.39	–4.06±4.16	–1.88±2.02	4.85±4.06
P content	Control	digenic	digenic	–39.52±4.76**	–43.59±6.94**	digenic	digenic
	AMF	digenic	digenic	–42.24±5.77**	–49.42±11.56**	digenic	digenic
P uptake	Control	0.70±0.77	–2.23±1.56	–0.69±0.65	–3.62±1.31**	0.09±0.85	0.16±1.74
	AMF	1.36±0.91	–2.33±1.58	–0.66±0.87	–4.34±1.75**	–0.07±0.80	1.07±1.28
	AMF+*Azc*	1.44±0.76	–2.67±1.49	–0.68±0.67	–3.23±1.33*	0.66±0.97	0.84±1.74
P response (%)	AMF	–0.52±2.87	–1.27±4.70	2.22±2.06	–2.64±2.83	1.49±1.80	1.50±4.53
	AMF+*Azc*	–0.32±3.14	2.02±6.08	2.93±2.66	0.79±5.60	3.13±2.30	0.02±4.00
P use index (%)	AMF	0.38±2.25	–1.74±5.71	1.68±2.25	–1.94±3.68	1.32±2.03	.31±4.50
	AMF+*Azc*	–1.45±2.49	1.82±4.53	3.74±2.65	–0.58±5.08	2.62±1.47	0.73±3.25

d = additive gene effects; h = dominance gene effects

Singh et al. (2007) reported significant variation for AMF infection in the roots for the same experimental population, ranging from 19.07% (WH 147 × WH 533) to 19.80% (WH 533 × Raj 3077) in the control, from 30.63% (WH 147 × Raj 3077) to 37.17% (WH 533 × Raj 3077) in the AMF treatment and from 39.43% (WH 147 × Raj 3077) to 41.52% (WH 533 × Raj 3077) in the AMF+*Azc* treatment.

Azotobacter excretes phytohormones, which improve plant growth, while AMF solubilizes P from surrounding areas and makes it available to the plant. Dual inoculation with efficient strains of *Azc* and *Glomus fasciculatum* in responsive wheat genotypes adapted to low input stress conditions could be profitably used to enhance nutrient use efficiency and hence to maximize wheat production. Intense AMF infection of the roots is important even at moderate nutrient deficiency during early plant growth, when the roots are too small to supply the high demand for minerals for shoot growth. In this context, the selection of potent recombinants in crosses involving wheat variety WH 147, suitable for medium fertility and water deficit conditions, holds great promise for the development of high-yielding genotypes responsive to bio-inoculants for stress-prone, low-input conditions. Thus, pedigree selection in the crosses WH 147 × WH 533 and WH 147 × Raj 3077 could be effective for breeding pure lines of wheat for sustainable agriculture (low-input genotypes responsive to biofertilizers such as AMF and *Azotobacter*). Genotypes developed from such crosses will make better use of applied nutrients, especially N and P, and will be suitable for sustainable wheat production in soils having high pH, where P fixation problems are to be expected.

Table 3

Estimation of digenic interactions in three wheat crosses for nitrogen (N) and phosphorus (P) contents (ppm), N uptake (mg/plant), N response (%) and N use index (%) in different bio-inoculation treatments

Treatment	Cross	d	h	i	j	l	E
N content							
Control	WH 147 × WH533	133.90±4.73**	–6232.30±19.92**	–384.20±12.63**	848.00±10.48**	5525.40±15.70**	D
	WH 533 × Raj 3077	578.35±4.57**	7880.95±20.19**	2957.60±13.13**	–2774.30±10.06**	–5959.30±15.60**	D
	WH 147 × Raj 3077	802.30±4.13**	7551.80±17.26**	1698.60±11.06**	–1402.80±8.91	–6473.60±13.54**	D
AMF	WH 147 × WH533	43.15±3.57**	2292.15±18.39**	1281.60±12.31**	353.70±8.30**	2884.10±13.95**	D
	WH 533 × Raj 3077	609.55±4.27**	–1422.45±18.25**	171.20±11.73**	–1007.10±9.32**	1775.10±14.31**	D
AMF+*Azc*	WH 147 × WH533	509.45±4.49**	–7132.15±18.33**	–2732.00±11.59**	1576.70±9.72**	3536.10±14.45**	D
	WH 533 × Raj 3077	683.35±4.46**	10155.45±19.48**	4262.20±12.57**	–872.10±9.90**	–6689.10±15.12**	D
	WH 147 × Raj 3077	486.40±3.80**	–3100.60±17.74**	–670.80±11.71**	–508.00±8.46**	1660.40±13.62**	D
N uptake							
Control	WH 147 × WH533	19.76±1.30**	–236.54±5.58**	–65.60±3.81**	–21.52±2.93**	181.92±4.60**	D
	WH 533 × Raj 3077	9.57±1.26**	594.07±5.45**	237.88±3.58**	18.81±2.64**	–394.37±4.19**	D
	WH 147 × Raj 3077	26.45±1.38**	145.18±5.91**	23.72±3.82**	–38.95±2.98**	–150.07±4.59**	D
AMF	WH 147 × WH533	7.27±1.38**	78.38±5.80**	33.28±3.81**	–24.55±2.82**	–56.63±4.50**	D
	WH 533 × Raj 3077	18.41±1.26**	534.60±5.46**	199.50±3.56**	–28.53±2.70**	–393.47±4.23**	D
	WH 147 × Raj 3077	32.95±1.32**	139.45±5.34**	25.70±3.49**	–54.96±2.66**	–120.78±4.15**	D
AMF+*Azc*	WH 147 × WH533	6.00±1.37**	72.30±5.78**	47.00±3.77**	–18.60±2.86**	–33.40±14.51**	D
	WH 533 × Raj 3077	14.60±1.31**	495.35±5.52**	193.68±3.58**	–31.01±2.77**	–358.37±4.28**	D
	WH 147 × Raj 3077	25.55±1.48**	–70.29±5.94**	–57.30±3.85**	–33.32±3.01**	–3.46±4.64**	–
N response							
AMF	WH 147 × WH533	–4.45±1.34**	–143.12±6.92**	49.00±4.60**	–8.13±3.18**	98.11±5.29**	D
	WH 533 × Raj 3077	4.64±1.54**	–66.49±6.60**	–35.14±4.12**	–10.35±3.55**	29.09±5.25**	D
AMF+*Azc*	WH 147 × WH 533	–6.83±1.81**	149.74±7.61**	52.74±4.93**	–2.07±3.83**	–98.19±45.92**	D
N use index							
AMF	WH 147 × WH 533	–2.88±1.35**	82.67±5.90**	27.10±3.80**	–0.93±3.00**	–59.29±4.68**	D
P content							
Control	WH 147 × WH533	31.08±2.31**	88.88±9.45**	118.86±5.87**	–43.86±5.13**	142.68±7.58**	C
	WH 147 × Raj 3077	5.64±2.26*	–286.63±10.25**	–119.92±6.53**	–91.28±5.32**	167.14±8.01**	D
AMF	WH 147 × WH533	34.56±2.21**	797.84±9.95**	330.70±6.33**	–88.54±5.15**	–354.72±7.94**	D
	WH 147 × Raj 3077	14.27±2.26**	–144.06±10.07**	–67.84±6.65**	115.53±4.82**	66.39±7.71**	D
AMF+*Azc*	WH 147 × WH533	36.97±2.26**	486.73±10.05**	221.66±6.37**	–149.64±5.25**	–191.38±8.06**	D
	WH 533 × Raj 3077	62.43±2.42**	10.52±10.29	–11.90±6.61	–86.04±5.27**	–79.32±8.03**	D
	WH 147 × Raj 3077	7.98±2.34**	–282.08±8.13**	–138.06±4.91**	158.90±4.65**	141.72±6.58**	D

d = additive gene effects; h = dominance gene effects; i = additive × additive interactions; j = additive × dominance interactions; l = dominance × dominance interactions; E = epistasis, D = duplicate epistasis, C = complementary epistasis

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NITROGEN MITIGATES EFFECT OF SALINITY ON PLANT WATER RELATIONS AND PHOTOSYNTHESIS IN INDIAN MUSTARD (*Brassica juncea*)

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The interaction between salinity (8 and 12 dS m^{-1}) and three levels (40, 80 and 120 kg ha^{-1}) of different forms of nitrogen (NO_3^-, NH_4^+ and $NO_3^- + NH_4^+$) were studied in *Brassica juncea* cv. RH-30. The plants were salinized with 8 and 12 dS m^{-1} at 35 and 55 days after sowing. The relative water content (RWC), water potential (ψ_w) and osmotic potential (ψ_s) exhibited a marked decline under salinity stress. The application of the combined form ($NO_3^- + NH_4^+$) of nitrogen (120 kg ha^{-1}) considerably improved the water status and mitigated the adverse effect of salinity on growth. The salinity-induced osmotic effect led to stomatal closure and caused a substantial reduction in net photosynthetic rate (P_N), stomatal conductance (g_s) and transpiration rate (E) at the pre-flowering and flowering stages (45 and 65 DAS). Salinity effects were considerably moderated by additional nitrogen supply, which varied with the source of nitrogen, the level of salinity/fertilizer and the stage of plant growth. The inhibition in photosynthesis was relatively greater in ammonium-fed (NH_4^+) than in nitrate-fed (NO_3^-) plants, while the transpiration rate was relatively lower in nitrate-fed plants grown either with or without saline water irrigation. The nitrate form of nitrogen @ 120 kg ha^{-1} proved best in alleviating the adverse effect of salinity on photosynthesis and transpiration at both the growth stages.

Key words: water relations, photosynthesis, salinity, nitrogen source, *Brassica juncea*

Introduction

In warm dry areas the salt concentration increases in the upper soil layer due to high water losses, which exceed precipitation (Ebert et al., 2002). Overcoming salt stress is a major issue in these regions to ensure agricultural sustainability and crop production. Salinity is a major abiotic stress reducing the yield of a wide variety of crops all over the world (Tester and Davenport, 2003). In the semi-arid parts of India, the growth of Indian mustard is often restricted as the groundwater used for irrigation is mostly moderately to highly saline. A better understanding of how salinity affects physiological processes is important if such water is to be used efficiently. Nitrogen contributes substantially to plant growth and is directly related to crop yield potential. Nitrate (NO_3^-) and ammonium (NH_4^+) are the most abundant N sources for higher plants and their availability usually constitutes a limiting factor for plant growth (Frechilla et al., 2001; Nathawat et al., 2005; 2007). Salt stress interferes with nitrate uptake in many plants species due to the Cl^- content (Khan and Srivastava, 1998; Abdelgadir et al., 2005). The nitrogen source may affect the growth and productivity of barley (Ali et al., 2001) and wheat (Cramer and Lewis, 1993). However, most studies have been conducted in the absence of salinity, and the interaction between N source and water status has been given little attention. Thus, studies on the combined effect of these growing conditions on photosynthesis and water status are generally scarce and completely lacking for oil seeds. This paper reports on the joint effect of salinity and nitrogen forms at two growth stages (pre-flowering and flowering) on the water status and gas exchange parameters in plants.

Materials and methods

Plant materials and treatments

The experiment was conducted during October 1999 and 2000 in the pot house of the College of Basic Sciences and Humanities, CCS HAU, Hisar. Mustard plants (*Brassica juncea* cv. RH-30) were raised in pots, each of which was lined with polythene bags and filled with 6 kg of dune sand. Before seed sowing, the pots were supplied with nitrogen through different nitrogen sources, $Ca(NO_3)_2$, $(NH_4)_2SO_4$ and a combination of both at three levels, i.e. 40 mg kg^{-1}, 80 mg kg^{-1} and 120 mg kg^{-1} dune sand (equivalent to 40, 80 and 120 kg ha^{-1}). Balanced doses of PKS were given at the time of pot filling. After the application of fertilizers, the pots were saturated with water and allowed to settle over night. Sowing was carried out at the field capacity of dune sand. Ten seeds were sown in each pot at a uniform depth (1.5 to 2.0 cm) and distance. Fifteen days after sowing, thinning was done and three plants of uniform size were maintained per pot. Each pot was supplied with an equal quantity of N-free nutrient solution at regular intervals of 15 days. The desired salinity levels (ECe 0, 8 and 12 dS m^{-1}) were obtained by adding Cl and SO_4 salts of Na, Ca and Mg. For each ECe level, the requisite amount of salts per litre was added to the pots on a soil saturation basis at 35 DAS (Stage I) or 55 DAS (Stage II). A non-saline control was maintained separately. Sampling was done 10 days after applying saline water irrigation, i.e. 45 and 65 DAS (corresponding to the pre-flowering and flowering stages). Three replications were maintained for each treatment during the whole course of the experiment. The total plant dry weight was recorded at 45 and 65 DAS, while plant height was measured at harvest (Stages I and II). The chlorophyll and carotenoid contents were estimated according to the method of Hiscox and Israelstam (1979) using dimethyl sulphoxide (DMSO) as solvent.

Water relations

The relative water content (RWC) was determined by weighing the 3rd leaf from the top and floating it on de-ionized water for 6 h at constant temperature in diffused light. When the leaf became fully turgid, it was re-weighed, after which it was dried and the dry weight was determined. The RWC was calculated by the following formula (Barrs and Weatherley, 1962):

$$\text{RWC (\%)} = \frac{\text{Leaf fresh weight} - \text{leaf dry weight}}{\text{Leaf fully turgid weight} - \text{leaf dry weight}} \times 100$$

Leaf water potential (ψ_w) was determined from the third fully expanded leaf in a pressure chamber (Model-3000 series, Plant Water Status Console, Soil Moisture Equipment Corp., Santa Barbara, California, USA) using the technique introduced by Scholander et al. (1965). The values of water potential were expressed as –MPa. Simultaneously, adjacent leaves were harvested and frozen in liquid N_2 for osmotic potential (ψ_s) measurements. These frozen leaves were thawed and the osmotic potential was determined for the expressed solution after measuring sap osmolarity with a model 5100-B vapour pressure osmometer (Wescor Inc., Logan, Utah, USA). A standard curve was prepared with the help of sodium chloride solution. The values of osmotic potential of the leaf sap were recorded with the help of the standard curve and expressed in –MPa.

Leaf gas exchange characteristics

Various leaf gas exchange parameters such as net photosynthetic rate (μmol m^{-2} s^{-1}), transpiration rate (mmol m^{-2} s^{-1}) and stomatal conductance (mmol m^{-2} s^{-1}) were measured separately on the upper and lower surfaces of fully expanded leaves using a portable infrared gas analyzer (PP system Model CIRAS-1). These measurements were made on the third leaves between 9.30 to 11.00 A.M.

Statistical analysis of data

The statistical evaluation of the data was done for a factorial completely randomized design [3 salinity levels × 3 nitrogen forms × 3 nitrogen levels with 3 replicates] (Raghavarao, 1983).

Results

Effect of salinity and nitrogen source on growth

The total dry weight per plant and the plant height decreased at higher salinity levels from 8 to 12 dS m^{-1} over non-saline plants in both the pre-flowering and flowering stages. However, the plants generally maintained the highest total dry weight per plant and greatest plant height when grown with the combined (nitrate + ammonium) form N and the lowest with NH_4^+-N under saline conditions (Tables 1 and 2). Table 1 indicates that the percentage reduction in total dry weight per plant (34.70% and 47.76%) was maximum with NH_4^+-N and minimum (17.12% and 26.34%) with the combined form of nitrogen under 8 to 12 dS m^{-1} salinity compared with non-saline plants at the pre-flowering stage. A similar trend was observed at the flowering stage, the corresponding figures being 28.28% and 39.23% for NH_4^+-N and 14.51% and 21.72% for the combined form of nitrogen (Table 1). At the pre-flowering stage the total dry weight per plant was consistently greater with the highest dose of nitrogen (120 kg ha^{-1}) than with the lowest dose (40 kg ha^{-1}), irrespective of the N source, but the interactive effect between salinity and different levels of nitrogen sources was non-significant. Similarly, at the flowering stage the highest level of nitrogen application led to the greatest alleviation of the deleterious effect of salinity on total dry weight except for the highest level of the ammoniacal form (Table 1).

Table 1

Effect of nitrogen source, levels and their interaction with salinity on total plant dry weight (g) in *Brassica juncea* cv RH-30

Nitrogen sources	Nitrogen levels (kg ha^{-1})	Salinity levels (dS m^{-1})							
		0	8	12	Mean	0	8	12	Mean
		Pre-flowering stage (45 DAS)				Flowering stage (65 DAS)			
Nitrate form (NO$_3^-$)	40	1.631	1.205	1.038	1.291	2.418	1.903	1.464	1.928
	80	2.124	1.715	1.519	1.786	3.154	2.565	2.384	2.701
	120	2.322	2.005	1.769	2.032	3.78	3.335	3.005	3.373
	Mean	2.026	1.642	1.442	1.702	3.118	2.601	2.284	2.668
Ammoniacal form (NH$_4^+$)	40	1.242	0.745	0.588	0.859	1.762	1.272	1.06	1.374
	80	1.557	1.071	0.889	1.173	2.404	1.781	1.522	1.902
	120	1.153	0.763	0.587	0.834	2.021	1.406	1.195	1.541
	Mean	1.317	0.86	0.688	0.955	2.072	1.486	1.259	1.606
Combined form (NO$_3^-$ + NH$_4^+$)	40	1.553	1.111	1.012	1.225	2.49	2.006	1.679	2.058
	80	2.477	2.002	1.793	2.091	3.327	2.809	2.625	2.92
	120	2.471	2.275	1.982	2.243	3.958	3.543	3.35	3.617
	Mean	2.167	1.796	1.596	1.853	3.259	2.786	2.551	2.865
Overall mean		1.837	1.432	1.242		2.816	2.291	2.031	

C.D. ($P<0.05$) for NS/NL/SL = 0.03 C.D. ($P<0.05$) for NS/NL/SL = 0.027
C.D. ($P<0.05$) for NS×NL, NL×SL & NS×SL = 0.051 C.D. ($P<0.05$) for NS×NL, NL×SL & NS×SL = 0.046
C.D. ($P<0.05$) for NS×NL×SL = ns C.D. ($P<0.05$) for NS×NL×SL = 0.08
NS = Nitrogen source; NL = Nitrogen level; SL = Salinity level; ns = Non-significant

Table 2

Effect of nitrogen source, levels and their interaction with salinity on plant height (cm) in *Brassica juncea* cv. RH-30 at harvest

Nitrogen sources	Nitrogen levels (kg ha^{-1})	Salinity levels (dS m^{-1})							
		0	8	12	Mean	0	8	12	Mean
		Pre-flowering stage (45 DAS)				Flowering stage (65 DAS)			
Nitrate form (NO$_3^-$)	40	99.1	84.5	75.6	86.4	94.6	78.1	69.1	80.6
	80	113.0	99.4	91.5	101.3	111.8	94.3	85.6	97.2
	120	119.0	107.7	99.3	108.6	115.4	101.2	93.4	103.3
	Mean	110.3	97.2	88.8	98.7	107.2	91.2	82.7	93.7
Ammoniacal form (NH$_4^+$)	40	94.7	84.8	70.9	83.4	89	70.1	61.4	73.5
	80	113.9	95.0	87.8	98.9	108.1	91.7	83.1	94.3
	120	105.1	85.2	79.3	89.8	99.2	79.5	73.5	84
	Mean	104.5	88.3	79.3	90.7	98.7	80.4	72.6	83.9
Combined form (NO$_3^-$ + NH$_4^+$)	40	110.3	96.3	86.1	97.5	106.4	87.1	79.3	90.9
	80	127.1	103.3	101.7	110.7	122.6	104.8	95.7	107.7
	120	128.4	114.5	106.2	116.3	126.3	110.5	101.5	112.7
	Mean	121.9	104.6	98	108.1	118.4	100.8	92.1	
Overall mean		112.0	96.0	88.0		108.1	90.8	82.4	

C.D. ($P<0.05$) for NS/NL/SL = 2.637 C.D. ($P<0.05$) for NS/NL/SL = 2.661
C.D. ($P<0.05$) for NS×NL, NL×SL & NS×SL = 4.566 C.D. ($P<0.05$) for NS×NL, NL×SL & NS×SL = 4.611
C.D. ($P<0.05$) for NS×NL×SL = 7.908 C.D. ($P<0.05$) for NS×NL×SL = ns
NS = Nitrogen source; NL = Nitrogen level; SL = Salinity level; ns = Non-significant

Plant height decreased with higher salinity levels (8 to 12 dS m^{-1}) compared with non-saline plants at harvest (Stages I and II). The detrimental effect of salinity was more pronounced in ammonium-fed plants than for nitrate or the combined form of N. The decline in plant height was maximum (15.50 and 24.11%) with the ammoniacal form and minimum (14.19 and 19.60%) with the combined form of N at the two levels of salinity at Stage I, while these figures were 18.54 and 26.44% for NH_4^+-N and 14.86 and 22.21% for the combined form of nitrogen at Stage II. The reduction in plant height induced by salinity was mitigated by the application of the highest level of N through different sources. However, the combined form of N (120 kg ha^{-1}) counteracted the adverse effect of salinity to a greater extent than the other two forms at both sampling stages (Table 2).

Effect of salinity and nitrogen source on photosynthetic pigments

A decline in photosynthetic pigments (chlorophyll and carotenoid) was observed under salinity (8 and 12 dS m^{-1}) compared with the non-saline control at the pre-flowering and flowering stages (Tables 3 and 4). At pre-flowering this reduction in chlorophyll content under saline conditions was maximum (25.32 and 38.73%) for the ammoniacal form and minimum (21.99 and 33.73%) for the combined form of nitrogen over the control plants. In the same way, the maximum (18.69 and 35.33%) and minimum (14.45 and 25.14%) reductions were observed with the ammoniacal and combined form, respectively, compared to non-saline plants at the flowering stage (Table 3). Table 4 indicates that under saline conditions the reduction in carotenoid content was highest (18.23 and 16.35%) with the ammoniacal form and lowest (11.93 and 15.34%) with the combined form of nitrogen at the pre-flowering stage. Similarly, at the flowering stage the decline was maximum (17.15 and 21.30%) for NH_4^+-N and minimum (11.76 and 17.64%) for the combined form of nitrogen as compared to the control plants. Irrespective of the nitrogen source the higher levels of nitrogen (80 and 120 kg ha^{-1}) increased the chlorophyll and carotenoid contents of the leaves significantly as compared to the lowest level of nitrogen (40 kg ha^{-1}) at both sampling stages, but the interactive effect of salinity and different levels of nitrogen sources was non-significant at both the pre-flowering and flowering stages (Tables 3 and 4).

Effect of salinity and nitrogen source on water relations

The reduction in RWC in leaves under saline conditions (8 and 12 dS m^{-1}) was greatest (17.58 and 27.95%) with the ammoniacal form and smallest (13.49 and 22.16%) with the combined form of N at the pre-flowering stage (45 DAS). Higher levels (80 and 120 kg ha^{-1}) of all the nitrogen sources (NO_3^-, NH_4^+ and NO_3^- + NH_4^+) showed a significant increase in RWC over the lowest level of nitrogen (40 kg ha^{-1}) at both the sampling dates, but the interaction between salinity and different levels of nitrogen sources was non-significant (Fig. 1A).

Table 3

Effect of nitrogen source, level and their interaction with salinity on total chlorophyll content (mg g^{-1} dry weight) in leaves of *Brassica juncea* cv. RH-30

Nitrogen sources	Nitrogen levels (kg ha^{-1})	Salinity levels (dS m^{-1})							
		0	8	12	Mean	0	8	12	Mean
		Pre-flowering stage (45 DAS)				Flowering stage (65 DAS)			
Nitrate form (NO$_3^-$)	40	18.89	13.64	10.72	14.42	14.29	11.80	8.91	11.67
	80	19.23	14.71	11.92	15.29	16.56	14.02	12.17	14.25
	120	19.43	15.76	14.25	16.48	18.35	16.39	14.70	16.48
	Mean	19.18	14.70	12.30	15.39	16.40	14.07	11.93	14.13
Ammoniacal form (NH$_4^+$)	40	14.33	10.21	7.75	10.76	13.51	11.02	7.95	10.83
	80	15.73	11.92	9.33	12.32	14.25	11.94	9.63	11.94
	120	16.25	12.47	11.29	13.34	14.61	11.49	9.82	11.97
	Mean	15.44	11.53	9.46	12.14	14.12	11.48	9.13	11.58
Combined form (NO$_3^-$ + NH$_4^+$)	40	19.13	14.31	11.72	15.05	15.80	13.22	10.79	13.27
	80	20.79	16.32	13.56	16.89	16.19	13.93	12.10	14.07
	120	22.43	18.02	16.04	18.83	18.23	15.82	14.71	16.25
	Mean	20.78	16.21	13.77	16.92	16.74	14.32	12.53	14.53
Overall mean		18.49	14.15	11.84		15.75	13.29	11.20	

C.D. ($P<0.05$) for NS/NL/SL = 0.208 C.D. ($P<0.05$) for NS/NL/SL = 0.187
C.D. ($P<0.05$) for NS×NL, NL×SL & NS×SL = 0.36 C.D. ($P<0.05$) for NS×NL, NL×SL & NS×SL = 0.323
C.D. ($P<0.05$) for NS×NL×SL = ns C.D. ($P<0.05$) for NS×NL×SL= ns
NS = Nitrogen source; NL = Nitrogen level; SL = Salinity level; ns = Non-significant

Table 4

Effect of nitrogen source, level and their interaction with salinity on carotenoid content (mg g^{-1} dry weight) in leaves of *Brassica juncea* cv. RH-30

Nitrogen sources	Nitrogen levels (kg ha^{-1})	Salinity levels (dS m^{-1})							
		0	8	12	Mean	0	8	12	Mean
		Pre-flowering stage (45 DAS)				Flowering stage (65 DAS)			
Nitrate form (NO$_3^-$)	40	1.62	1.38	1.29	1.43	1.75	1.42	1.32	1.50
	80	1.74	1.51	1.49	1.58	1.86	1.64	1.53	1.68
	120	1.82	1.60	1.61	1.67	1.97	1.83	1.72	1.84
	Mean	1.72	1.50	1.46	1.56	1.86	1.63	1.52	1.67
Ammoniacal form (NH$_4^+$)	40	1.65	1.28	1.57	1.50	1.73	1.37	1.29	1.46
	80	1.67	1.34	1.28	1.43	1.79	1.49	1.43	1.57
	120	1.46	1.30	1.13	1.29	1.55	1.33	1.26	1.38
	Mean	1.59	1.30	1.33	1.41	1.69	1.40	1.33	1.47
Combined form (NO$_3^-$ + NH$_4^+$)	40	1.63	1.39	1.26	1.43	1.76	1.44	1.33	1.51
	80	1.75	1.55	1.51	1.61	1.88	1.68	1.54	1.70
	120	1.89	1.71	1.68	1.76	1.98	1.84	1.74	1.85
	Mean	1.76	1.55	1.49	1.60	1.87	1.65	1.54	1.69
Overall mean		1.69	1.45	1.42		1.81	1.56	1.46	

C.D. ($P<0.05$) for NS/NL/SL = 0.071 C.D. ($P<0.05$) for NS/NL/SL = 0.025
C.D. ($P<0.05$) for NS×NL, NL×SL & NS×SL = 0.124 C.D. ($P<0.05$) for NS×NL, NL×SL & NS×SL = 0.043
C.D. ($P<0.05$) for NS×NL×SL = ns C.D. ($P<0.05$) for NS×NL×SL = ns
NS = Nitrogen source; NL = Nitrogen level; SL = Salinity level; ns = Non-significant

The decline in water potential under saline conditions (8 and 12 dS m^{-1}) was most pronounced (25.08 and 32.33%) for the NH_4^+-N and lowest (12.21 and 19.36%) for the combined form of N, over non-saline conditions at the pre-flowering stage. In the same way, at the flowering stage these reductions were greatest (27.06 and 32.41%) with ammoniacal and smallest (10.33 and 20.48%) for the combined form of N at the 8 and 12 dS m^{-1} levels of salinity. The application of N (120 kg ha^{-1}) in combined form caused a smaller decrease (6.25 and 12.50%) in water potential at salinity levels of 8 and 12 dS m^{-1} at both sampling stages (Fig. 1B).

The osmotic potential in the leaves declined appreciably with salinity at both the pre-flowering and flowering stages. Under salt stress (8 and 12 dS m^{-1}) the highest percentage reduction (25.31 and 41.89%) was observed for NH_4^+-N and the lowest (13.62 and 27.60%) for the combined form of N at the pre-flowering stage. At the flowering stage, too, the combined form of nitrogen resulted in the least reduction (18.06 and 25.73%) in osmotic potential, whilst the greatest reduction (25.21 and 44.85%) was observed with the ammoniacal form of nitrogen. The highest dose of the combined form of N (120 kg ha^{-1}) caused less reduction in the osmotic potential of the leaves than the lowest N dose (40 kg ha^{-1}) under saline conditions. In contrast, the highest rate of the ammoniacal form of N (120 kg ha^{-1}) resulted in the greatest upsurge in the osmotic potential of the leaves at both sampling stages (Fig. 1C).

Effect of salinity and nitrogen source on photosynthesis

The reduction in the photosynthetic rate was relatively lower in nitrate-fed plants grown either with or without saline water irrigation as compared to the control plants. These reductions under salinity were maximum (27.81 and 39.20%) and minimum (26.76 and 32.70%) with the application of the ammoniacal and nitrate form of nitrogen, respectively, as compared to control plants at the pre-flowering stage. Correspondingly, plants treated with the ammoniacal form of nitrogen showed the greatest reduction (20.67 and 36.23%) in the photosynthetic rate and the nitrate form the smallest reduction (13.92 and 25.55%) as compared to non-saline plants at the flowering stage (Fig. 2A). The highest level of nitrogen (120 kg ha^{-1}) in nitrate form exhibited the smallest reduction (13.92 and 27.10%) in the photosynthetic rate at salinity levels of 8 and 12 dS m^{-1}, respectively, compared to non-saline plants. This reduction was maximum (18.99 and 39.94%) at the lowest level of nitrogen application at 45 DAS. Furthermore, increasing the level of nitrogen (80 and 120 kg ha^{-1}), irrespective of the source of nitrogen, gave a considerably higher photosynthetic rate in the leaves, except for the application of the ammoniacal form at 120 kg ha^{-1}, compared to the lowest dose of nitrogen, i.e. 40 kg ha^{-1}. The interactive effect between salinity and different levels of nitrogen sources was found to be non-significant at 65 DAS (Fig. 2A).

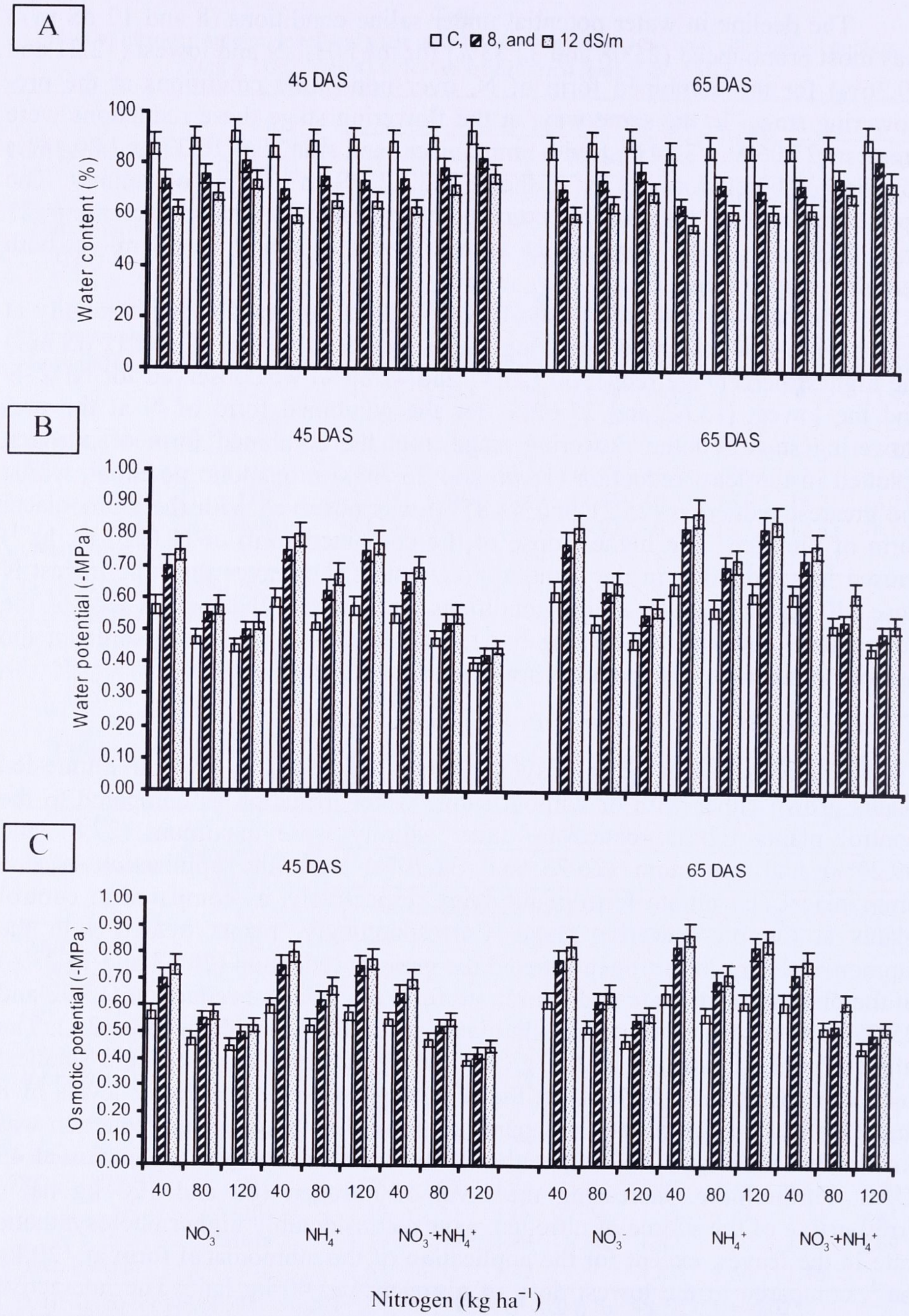

Fig. 1. Effect of nitrogen source, levels and their interaction with salinity on (A) water content (%), (B) Water potential (–MPa) and (C) osmotic potential (–MPa) in the leaves of *Brassica juncea* cv. RH-30

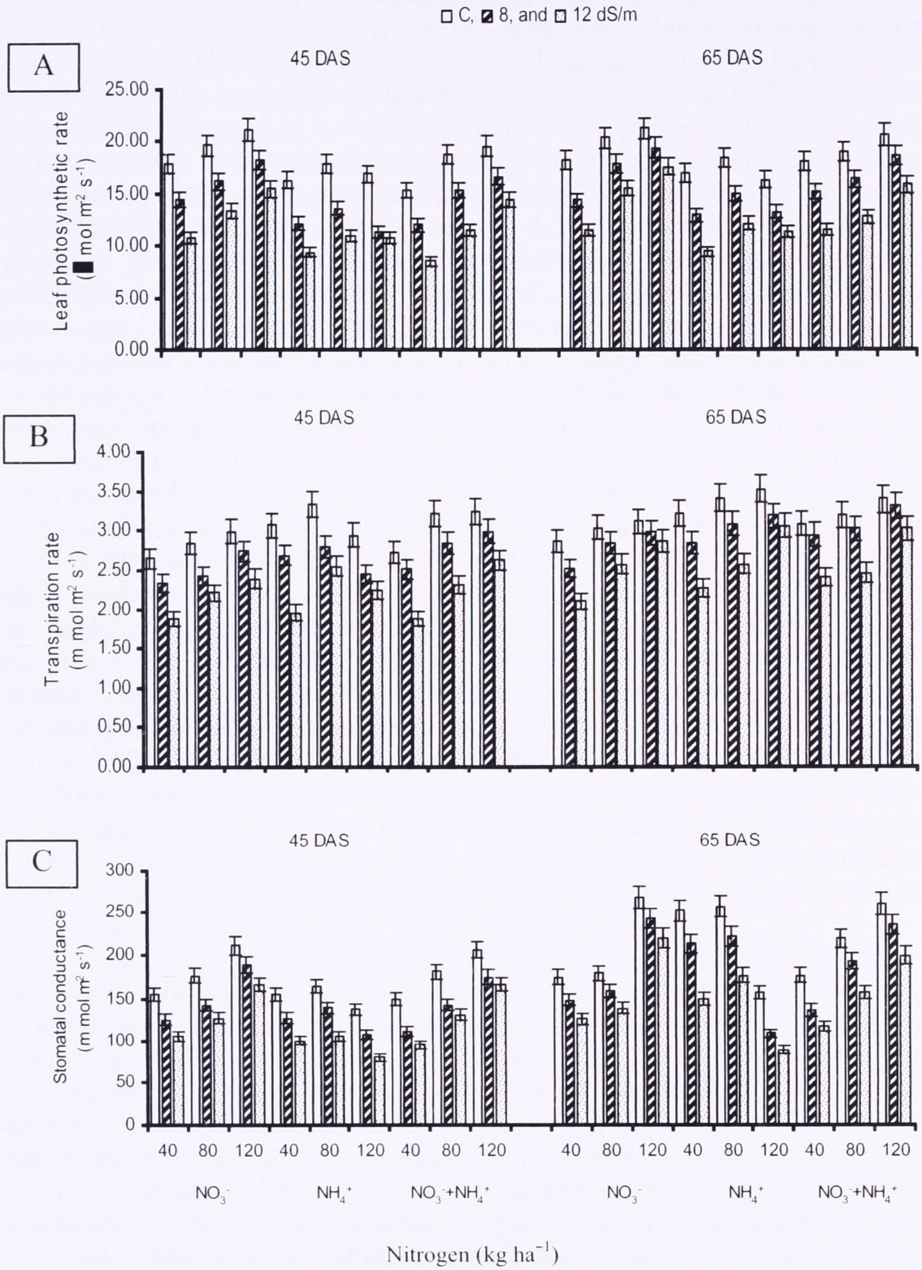

Fig. 2. Effect of nitrogen source, levels and their interaction with salinity on (A) leaf photosynthetic rate (□mol m^{-2} s^{-1}), (B) transpiration rate (mmol m^{-2} s^{-1}) and (C) stomatal conductance (mmol m^{-2} s^{-1}) in *Brassica juncea* cv. RH-30

Figure 2B illustrates that the transpiration rate decreased with increasing levels of salinity (8 and 12 dS m^{-1}). The reduction due to salinity was greatest (14.79 and 27.65% at 8 and 12 dS m^{-1}, respectively) for NH_4^+-N and smallest (11.66 and 23.67%) for NO_3^--N at the pre-flowering stage. Equally, treating plants with the ammoniacal form of nitrogen resulted in the maximum reduction (10.35 and 22.18%) in the transpiration rate, while the nitrate form of nitrogen led to a smaller reduction (7.64 and 16.61%) in comparison to non-saline plants at the flowering stage (Fig. 2B). A nitrogen dose of 120 kg ha^{-1} in nitrate form caused the least decrease (8.36 and 20.06%) in the transpiration rate at salinity levels of 8 and 12 dS m^{-1}, while this reduction was highest (12.07 and 29.05%) at the lowest level of the combined form of nitrogen, i.e. 40 kg ha^{-1} at 45 DAS. The highest level of nitrogen (120 kg ha^{-1}) in the nitrate form resulted in the least reduction (4.18 and 8.03%) in the transpiration rate at 65 DAS (Fig. 2B).

Stomatal conductance was found to decrease due to salinity stress, with the greatest reduction (18.45 and 37.85%) for plants treated with the ammoniacal form of nitrogen and the least reduction (15.86 and 26.56%) for the nitrate form, as compared to control plants at 45 DAS. The reduction at the two salinity levels was also highest (18.33 and 37.77%) for NH_4^+-N and lowest (11.68 and 22.25%) for NO_3^- -N at 65 DAS (Fig. 2C). N fertilization induced a significant rise in the stomatal conductance of stressed plants by improving the water status. The stomatal conductance was highest in plants treated with the nitrate form of nitrogen as compared to the ammoniacal form at both sampling stages. The least reduction in the stomatal conductance under saline conditions (10.54 and 21.57% at 45 DAS) was recorded at the highest rate of NO_3^- -N (120 kg ha^{-1}). At 65 DAS the least reduction (9.45 and 18.16%) in stomatal conductance was observed for the highest level of nitrate-N and the greatest reduction (15.26 and 28.05%) for the lowest level (Fig. 2C).

Discussion

Indian mustard is generally grown in arid and semi-arid regions where the groundwater used for irrigation is moderately to highly saline. Since nitrogen contributes substantially to plant growth, an understanding of the interactive effect of nitrogen forms and their combinations with a saline environment on mustard is an important practical aspect for plant growth and its relation to crop productivity. Total dry plant weight decreased at the pre-flowering and flowering stages under salinity stress. The decline in total plant dry weight was found to be greatest with the ammoniacal form and lowest with the combined form of nitrogen under saline conditions at both sampling stages (Table 1). Similar studies have shown that the dry plant weight decreased under salt stress (Abdelgadir et al., 2005). Salinity caused a clear reduction in plant biomass. The biomass production of ammonium-fed plants was lower than that of nitrate-fed plants (Rios-Gonzalez et al., 2002). The highest level of the combined form of nitrogen (120 kg ha^{-1}) significantly alleviated the deleterious effect of salt stress (8 dS m^{-1}) on total plant dry weight as compared to lower levels in the flowering stage (Table 1). It is interesting to note that the percentage reduction in dry matter production under salt stress was relatively less with the combined (NO_3^- + NH_4^+) form of nitrogen, as compared to the two individual forms. Among the different sources of N, the lowest dose (40 kg ha^{-1}) of NH_4^+-N exhibited the least ameliorating effect on growth parameters, while the highest dose (120 kg ha^{-1}) aggravated the deleterious effect of salinity (12 dS m^{-1}) at the flowering stage (Table 1). The reduction in dry matter under salinity is attributed to a decrease in metabolic activity. The impairment of the N-metabolism is one of the primary problems for plant growth under a saline environment (Nathawat et al., 2005). A decline in plant height was observed with salinity (8 and 12 dS m^{-1}) at both sampling stages, though the combined form of nitrogen (120 kg ha^{-1}) alleviated the deleterious effect of salinity on plant height at stage I (Table 2). Growth declined under saline stress but nitrate-fed plants were less sensitive to salinity than ammonium-fed plants. This different sensitivity was due mainly to the better maintenance of root growth in nitrate-fed plants (Frechilla et al., 2001).

The highest inhibitory effect of salinity (8 and 12 dS m^{-1}) on photosynthetic pigments (chlorophyll and carotenoids) was observed for the ammoniacal form and the lowest for the combined form of nitrogen at both the pre-flowering and flowering stages. The chlorophyll content of the leaves decreases in general under salt stress (Mishra et al., 2006). Significant increases in photosynthetic pigments were noticed with higher levels of nitrogen at both stages, suggesting that the adverse effect of salinity on chlorophyll and carotenoids was partially mitigated by applying the combined form of nitrogen at 120 kg ha^{-1} (Tables 3 and 4). In conformity with this, Garg et al. (1990) also reported that the levels of total chlorophyll declined under salt stress, but that nutritional improvement under both normal and saline conditions led to a higher concentration of chlorophyll.

Under saline conditions there was a substantial reduction in plant water status in terms of relative water content, water potential and osmotic potential. These parameters showed the greatest reduction in the ammoniacal form and the lowest reduction in the combined form of N as compared to non-saline plants at the pre-flowering and flowering stages (Fig. 1). N-deficiency markedly decreased the abundance of Hg-sensitive water channels (Carvajal et al., 1996), which could lower the steady-state water potential of leaves, preventing them from maintaining adequate turgor for growth (Radin and Boyer, 1982). The presence of salt in the soil solution decreased the osmotic potential of the soil, thereby resulting in water stress and making it difficult for the plants to absorb the water necessary for growth. Leaf water potential also decreased (Munns, 1993), although this decrease was accompanied by a decrease in leaf osmotic potential, so as to maintain the leaf turgor pressure of the salinised plants (Tattini et al., 1995).

It is evident from the data recorded under saline conditions that there was a decline in P_N, E and g_s as compared to the control plants. The reduction in the photosynthetic rate was relatively lower in nitrate-fed plants grown either with or without saline water irrigation compared to the ammoniacal form (Fig. 2A). Among the N forms, the increase in transpiration rate was greater for ammonium-N than for nitrate-N (Fig. 2B). A decrease in stomatal conductance was noted with increasing salinity level, showing a maximum decrease for the ammoniacal form and the least reduction for the nitrate form (Fig. 2C). Net CO_2 assimilation, stomatal conductance and transpiration rate were markedly decreased by the salt treatment (Khan et al., 1994; Tattini et al., 2002). NH_4^+-fed plants transpired more per unit leaf area and had smaller leaf mass than NO_3^--fed plants. Both facts suggest some sort of regulation of the water economy, when leaf area seems to be diminished by salinity-induced water stress and stomata continue losing water at higher rates. This phenomenon could be related to the importance of K^+ and its recirculation for N uptake and assimilation (Lips et al., 1987; Van Beusichem et al., 1988), hence the growth response and protective effect of K^+ on photosynthesis against the inhibitory effects of water stress.

Studies on the effect of improved soil fertility on RWC, water potential, osmotic potential, photosynthetic rate, transpiration rate and stomatal conductance firmly established the importance of a salinity-fertility interaction at the pre-flowering and flowering stages (Figs. 1 and 2). High values of RWC, ψ_w and ψ_s were maintained with the combined form in comparison to the ammoniacal form of N (Fig. 1). The application of N in the combined form (120 kg ha^{-1}) considerably improved the water status in terms of RWC and water potential (Fig. 1A, B). Nutritionally induced tolerance to salt stress was reported earlier in various crops (Garg et al., 1990; Khan et al., 1994). It was evident from the data that under saline conditions plants exhibited a decrease in the photosynthetic rate. The highest level (120 kg ha^{-1}) of NO_3^--N considerably improved the photosynthetic rate compared with non-saline plants, but the highest level of NH_4^+-N was found to have an inhibitory effect on the photosynthetic rate (Fig. 2A). The addition of N to plants subjected to salinity improved their growth and nitrogen-metabolizing enzymes, and thus their salt tolerance (Dubey and Pessarakli, 1995; Nathawat et al., 2005).

The transpiration and stomatal conductance decreased with increasing levels of salinity at the pre-flowering and flowering stage irrespective of the form of nitrogen (Fig. 2B, C). Among the different nitrogen forms, the transpiration rate and stomatal conductance were increased more by ammonium-N than by nitrate-N. According to Hsio and Lauchli (1986), plants showed a relatively higher transpiration rate under ammonium-N, which could be related to the depletion of other cations by the presence of an excessive concentration of ammonium (NH_4^+). This view was further strengthened in the highest ammonium-N treatment, where the plants exhibited a sharp increase in their transpiration rate (Khan et al., 1994). The nitrate form of nitrogen proved beneficial irrespective of the rate, while the ammoniacal form was beneficial up to 80 kg ha^{-1} and became inhibitory at 120 kg ha^{-1}. Besides this, the highest level of NO_3^--N (120 kg ha^{-1}) resulted in the smallest reduction in the transpiration rate and stomatal conductance under saline conditions as compared to non-saline plants (Fig. 2B, C). The present results are in accordance with other reports in which a reduction in photosynthesis was observed in plants grown in the presence of NaCl, which was attributed to stomatal resistance or to a reduction in the capacity of the photosynthetic machinery (Khan et al., 1994).

Conclusions

Plant water relations and gas exchange parameters were adversely affected by salt stress. The combined form of nitrogen (120 kg ha^{-1}) considerably improved the water status and mitigated the adverse effects of salinity. The inhibition of gas exchange parameters under salinity was partially restored with the use of different nitrogen sources. The nitrate form of nitrogen (120 kg ha^{-1}) proved better than the other two forms (combined and ammoniacal) in partially reviving the gas exchange parameters.

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EVALUATION OF THE RE-USE OF TREATED WASTEWATER FOR IRRIGATION

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Fresh water is considered one of the most important factors in expanding the cultivated area. In arid and semi-arid zones, water resources are scarce. Recently many scientists in different countries have concluded that the re-use of wastewater could help to solve water scarcity problems. An additional target is to protect the environment by reducing the pollution load with little or no risk to the plants, groundwater or human health. Therefore, the principal objective of the present study was to demonstrate the economic aspects of re-using secondary treated wastewater in irrigation, in order to make the best use of existing resources. Thus, field trials were established in a rotation of summer and winter crops during the 2000–2002 seasons to evaluate the effect of irrigation with secondary treated wastewater on the yield and quality of field crops compared with canal water. The present study discusses a part of this study, concerning the effect on the yield and quality of lentils and pearl millet. The experimental treatments for both crops were the same in both growing seasons, involving two water irrigation sources (secondary treated wastewater and canal water) and two fertilization treatments (application of recommended rates of chemical fertilizers and control without fertilizer application). The data demonstrated that crops irrigated with secondary treated wastewater performed equally well or significantly better than those irrigated with canal water. Heavy metal concentrations were very low, and had no influence on crop quality, determined as the chemical composition of lentil seeds and the dry forage yield of millet. Animal performance was also taken into consideration. The results indicated that the seed and biological yields of plants given wastewater in the absence of chemical fertilizers were nearly equal to those of plants given the recommended dose of chemical fertilizers, indicating that wastewater could provide an adequate amount of N, P and K to cover crop requirements at different growth stages.

Key words: wastewater, irrigation, heavy metal concentration

Introduction

The current water budget in Egypt shows that the annual water demand exceeds the available fresh water by 6 billion m^3/year (Abu-Zaid, 2006). Water use is rising due to the ambitious land reclamation programme, the growing population, steady rural development and urbanization plans and the expanding industrial sector. Therefore, it is essential to develop water resources in non-traditional ways.

During recent years, the management of wastewater has shifted from conventional disposal strategies to value-added products (Liang et al., 2003). The trials of Sudha Bansal and Kapoor (2000) demonstrated that the use of recycled wastewater effluent could supply nutrients to crops, while also improving the physical status and fertility of the soil.

Wastewater has been used to support agricultural production in many countries, such as the USA, Germany, India, Kuwait, Saudi Arabia, Oman, Jordan and Tunisia (Rowe and Abd-El Magid, 1995). The area of land irrigated with wastewater has increased significantly over the past two decades due to constraints on the water supply and increasing concerns over environmental implications.

In Egypt, the re-use of municipal wastewater for irrigation is not a new concept, but has been practised since 1911 on the sandy soil of El-Gabal El-Asfar farm, which consists of an area of 1260 ha. Situated 25 km north-east of Cairo, it is irrigated by wastewater from Cairo treatment plants and produces citrus, date palm and pecan nuts in addition to some field crops.

Several investigators indicated the beneficial role of wastewater in increasing crop yields with little or no risk to the plants, soil, groundwater or human health (Oron et al., 1991; 1992; Shatanawi and Fayyad, 1996; Vasquez-Montiel et al., 1996; Aissi et al. 1997; Palacios et al., 2000).

Therefore, the aim of this work was to evaluate the effect of treated wastewater on crop yield and quality on calcareous sandy soil under Egyptian conditions.

Materials and methods

Large-scale field trials were carried out over three years in a rotation begun in summer 2000 and the 2000/2001 winter season. The main objective of this study was to monitor the effect of using secondary treated wastewater compared with canal water in the presence or absence of the recommended dose of chemical fertilizers. An additional target was to offer a model for environmental improvement by opening up new ways of using treated wastewater in agriculture and consequently reducing the pollution load. The experimental site had sandy loam soil with high $CaCO_3$ (34.62%). The physical and chemical soil characteristics were studied according to the methods described by Burt (2004) and presented in Tables 1a, 1b and 2. The quality of the treated wastewater was analysed during the crop season (Table 3). The crops selected included a range of fodder and grain crops, according to WHO (1989). The present paper discusses the results achieved for the yield and quality of lentil and pearl millet. A surface flow irrigation system was used. The irrigation water was calculated from the number of tankers used during the growth period. The quantity of water was nearly equal for both sources of irrigation and was broadly in line with normal practice.

Chemical fertilizers (N, P and K) were applied according to the recommendations of the Egyptian Ministry of Agriculture for the experimental region. Ammonium nitrate (33.5% N), calcium superphosphate (15.5% P_2O_5) and potassium sulphate (48% K_2O) were applied at rates of 30, 100 and 60 kg/ha for lentil and 100, 150 and 80 kg/ha for millet.

Table 1a
Chemical properties of the soil sites

Depth (cm)	$CaCO_3$ (%)	Soluble anions (meq/l)				Soluble cations (meq/l)				EC (dS/m)	pH	SP (%)
		SO_4^{2-}	Cl^-	HCO_3^-	CO_3^{2-}	K^+	Na^+	Mg^{2+}	Ca^{2+}			
0–25	28.46	18.7	23.5	1.2	0	2.0	22.4	7.9	11.1	4.34	8.30	53.3
25–70	30.77	12.4	22.9	1.0	0	1.7	16.9	7.2	10.5	3.63	8.20	48.3
70–120	34.62	9.8	14.3	0.9	0	1.2	9.7	6.0	8.1	2.50	8.17	50.0

SP: Saturation percentage

Table 1b
Physical properties of the soil sites

Depth (cm)	FC (%)	WP (%)	AWC (%)	OM (%)	Particle size distribution (%)			
					Coarse sand	Fine sand	Silt	Clay
0–25	42.15	17.66	24.49	1.6	2.3	30.3	47.5	19.9
25–70	44.68	19.74	24.94	0.8	2.1	35.7	39.9	22.3
70–120	47.45	21.64	25.81	0.6	2.6	33.4	37.6	26.4

FC: Field water capacity; WP: Wilting point; AWC: Available water content; OM: Organic matter

Table 2
Available nutrients in the experimental soil

Depth (cm)	Organic matter (%)	Available nutrients (ppm)				
		Zn	Mn	Fe	P	Total N
0–25	1.7	1.5	3.9	3.1	2.5	4.17

Table 3
Chemical composition of treated wastewater

Constituent	Concentration	Constituent	Concentration
EC (dS/m)	3.10	CO^{2-} (meq/l)	1.10
pH	7.8	HCO^- (meq/l)	6.60
SAR	9.30	NH_4 (mg/l)	2.50
Na^+ (meq/l)	24.60	NO_3 (mg/l)	10.10
Ca^+ (meq/l)	1.50	P (mg/l)	8.50
Mg^{2+} (meq/l)	3.20	Mn (mg/l)	0.20
K^+ (meq/l)	1.80	Cu (mg/l)	1.10
Cl^- (meq/l)	62.00	Zn (mg/l)	0.80
SO^{2-} (meq/l)	35.00		

SAR: Sodium adsorption ratio = $[Na^+]/\sqrt{0.5([Ca^{2+}] + [Mg^{2+}])}$

The experimental design was a split plot design with four replications, with the water source in the main plot and the fertilization treatments assigned randomly in the sub-plots. Each experiment included four treatments, two water sources (secondary treated waste water, canal water) and two fertilization treatments (control without fertilizer and the recommended dose of NPK). The experimental area was ploughed twice, ridged and divided into 21 m^2 experimental units. Sowing was carried out at the recommended date for each crop and recommended agronomic practices were followed.

At harvest time, the two inner rows of each sub-plot were randomly sampled for determining the seed and biological yield in kg per plot, which was then converted to kg per hectare. Sub-samples of ten plants were taken to determine yield components, i.e. number of pods/plant and 1000-seed weight (g). Quality traits of lentil (fatty acid, crude protein and carbohydrate) were determined on absolutely dry seeds and samples of dry millet stem were taken to determine forage quality based on crude protein, fibre, ash, fat and soluble carbohydrate, according to A.O.A.C. (1984) methods. Micronutrients and heavy metals were determined after dry ash digestion according to Nakayama (1982).

The data obtained for each crop and each season were statistically analysed by analysis of variance according to Gomez and Gomez (1984). The trends of the three seasons were very similar. After a uniformity test, combined analyses for the three seasons were calculated as described by Cochran and Cox (1968). Treatment means were compared by the least significant difference (LSD) method.

Samples of treated wastewater were taken during the crop cycles and analysed according to Greenberg et al. (1992) for a range of agronomic, environmental and health parameters. Nutrient and heavy metal loading rates were calculated according to the irrigation quantities applied to each crop in order to assess the suitability of these wastewaters for re-use in the short and long term. Another objective was to determine the compliance of the wastewater with official Egyptian limit values.

Results and discussion

Effect on seed yield, yield components and lentil quality

Figures 1 and 3 clearly illustrate the yield response of lentil plants to irrigation with secondary treated wastewater on poorly fertile sandy soil. Irrigation with treated wastewater increased the seed yield of lentil by 8.83% compared with irrigation with canal water. This increment in seed yield could be attributed to the nutrient content in wastewater, which can supply part of the crop requirement for N and all the P and K. The data support the findings of Rowe and Abdel-Magid (1995), Vazquez-Montial et al. (1996), Palacios et al. (2000) and WRC (2001). Rowe and Abdel-Magid (1995) stated that the application of 25 mm wastewater was enough to supply 40–80% of corn requirements for N and all the P requirement. Data published by other authors suggested that the increase in yield as the result of irrigation with wastewater may be due to the enhancement of nutrient uptake and an improvement in the physical properties of the soil (Oron et al., 1991; 1992; Liang et al., 2003).

With regard to the application of the recommended dose of chemical fertilizers, the data indicated that the seed yield on plots irrigated with treated wastewater alone surpassed that on plots irrigated with canal water, but plants that received wastewater in the presence of chemical fertilizers gave the highest seed yield. This clearly confirmed that additional fertilizers are necessary to achieve an economic yield. Concerning seed quality, Figures 2 and 3 show that the fatty acid, crude protein and carbohydrate contents of lentil seeds were very similar regardless of whether the plants were irrigated with treated wastewater in the presence or absence of chemical fertilizers or with canal water.

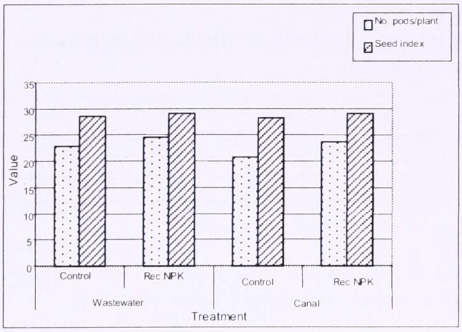

Fig. 1. Effect of source of water on yield and yield components of lentil

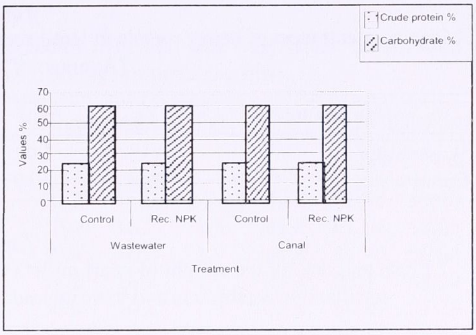

Fig. 2. Chemical composition of lentil seeds as affected by source of irrigation water

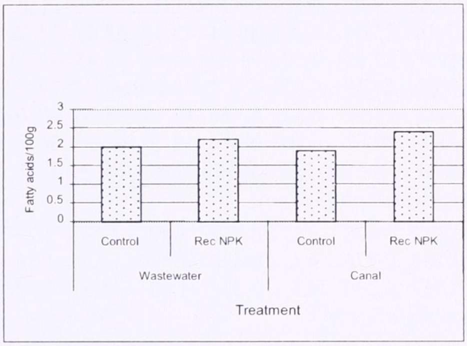

Fig. 3. Effect of water source on fatty acid concentration in lentil seeds

Rec NPK: Recommended rate of NPK

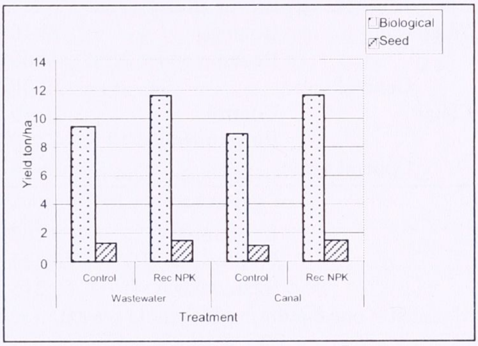

Fig. 4. Seed and biological yield of lentil as affected by source of irrigation water

The data presented in Table 4 clearly show that no consistent effect could be detected for the concentrations of heavy metals in seeds after irrigation with treated wastewater compared with canal water. Although the secondary treatment of wastewater removes up to 90% of the heavy metals, the wastewater still contains a small amount of contaminants. According to the data recorded, the concentrations are within the normal range and below the critical level. In addition, zinc and copper are essential trace elements, which are often deficient in Egyptian crops due to the high pH of the soil. This is confirmed by the deficiency of zinc and possibly copper seen in a number of crops from different locations.

Effect on fresh and dry weight and forage quality of pearl millet

The results obtained in Table 5 revealed that irrigation with treated wastewater increased the fresh and dry weight of both the 1st and 2nd cut and of the main cut of millet, the increments being 10.43%, 6.52% and 8.51% for fresh weight and 17.47%, 1.00% and 8.41% for dry weight for the first, second and main cut, respectively. In general, the data revealed that the forage yield of the main cut was higher than the total of two cuts; this may be due to the long period of growth for the main cut.

Table 4

Mean concentration of heavy metals in lentil seeds ($mg\ kg^{-1}$ dry seed) as affected by water source (Average of three seasons)

Water source	Zn	Cu	Cr	Cd	Pb	Ni
Wastewater	33.6	3.92	0.25	0.024	0.75	0.22
Canal water	28.8	3.19	0.17	0.022	0.36	0.18

Table 5

Fresh and dry forage weight of pearl millet (t/ha) as affected by irrigation with wastewater, fertilization treatments and their interactions (combined analysis of three seasons)

Water source	Fertilization treatment	1st cut		2nd cut		Total		Main cut	
		Fresh	Dry	Fresh	Dry	Fresh	Dry	Fresh	Dry
Wastewater	Control	34.16	7.48	25.55	6.48	59.71	13.96	68.67	35.28
	Recommended NPK	44.60	8.51	27.05	6.37	71.65	14.88	94.45	37.43
General mean		39.38	8.00	26.30	6.43	131.36	28.84	163.12	72.71
Canal	Control	29.19	5.82	23.36	6.32	52.55	12.14	68.26	30.76
	Recommended NPK	42.12	7.80	26.02	6.50	68.14	14.30	82.07	36.31
General mean		35.66	6.81	24.69	6.42	120.69	26.44	150.33	67.07
	LSD for								
	Irrigation (A)	0.287	0.342	0.328	n.s.	0.152	1.42	2.26	2.28
	Fertilization (B)	0.334	0.235	0.276	n.s.	0.423	1.53	1.32	1.16
	Interaction A×B	0.215	0.447	0.246	n.s.	0.214	1.62	1.25	1.24

Main cut = one cut during the whole season; n.s: non-significant

The application of chemical fertilizers in combination with both water sources caused a significant increase in the forage yield, confirming that additional fertilizers are necessary to achieve an economic yield, especially on poorly fertile soils. The present data are supported by the findings of Vyas et al. (1985) and Singh and Singh (1995). Concerning forage quality, the data in Table 6 indicated that in general, pearl millet gave good quality forage with a high content of crude protein (ranging from 7.5 to 8.9%) and digestible nutrients and a low content of fibre and lignin. The data also revealed that the water sources only caused significant differences in crude protein content, soluble carbohydrates and digestibility %. Animal performance tests did not reveal any difference between the forage obtained with the two water sources in terms of forage intake, which is a good indicator of forage quality. The data in Table 7 show that the heavy metal concentrations were within the normal ranges established by WHO (1989).

Conclusions

The present results highlight the importance of re-using secondary treated wastewater in irrigation in order to solve water scarcity problems in arid and semi-arid regions and to protect the environment by reducing the pollution load.

Table 6

Forage quality of the 2nd cut of pearl millet as affected by irrigation with wastewater, fertilization treatments and their interactions (Average of three seasons)

Water source	Fertilization treatment	CP	Fibre	Ash	Fat	SC	Digestibility (%)	
		(%)					1st cut	2nd cut
Wastewater	Control	7.8	30.2	10.0	10.8	49.8	62.35	53.90
	Recommended NPK	8.9	30.6	10.2	1.9	49.8	61.64	58.85
General mean		8.4	30.4	10.1	1.9	49.8	61.90	56.38
Canal	Control	7.5	31.2	10.2	1.7	49.7	60.42	54.60
	Recommended NPK	8.3	30.6	10.1	1.8	52.1	60.25	55.29
General mean		7.9	30.9	10.2	1.8	50.8	60.34	54.95
	LSD for							
	Irrigation (A)	0.34	n.s.	n.s.	n.s.	0.52	0.58	0.47
	Fertilization (B)	0.45	n.s.	n.s.	n.s.	0.84	0.34	0.88
	Interaction A×B	0.33	n.s.	n.s.	n.s.	0.43	0.28	0.67

CP: Crude protein; SC: Soluble carbohydrates; n.s: non-significant

Table 7

Mean concentration of heavy metals in two cuts of pearl millet as affected by water source (mg kg^{-1} dry weight, average of three seasons)

Water source	Zn	Cu	Cr	Cd	Pb	Ni
Wastewater	31.7	3.85	0.26	0.024	0.68	0.25
Canal water	29.8	3.22	0.14	0.020	0.38	0.16

It is also clear that crops irrigated with secondary treated wastewater performed equally well or significantly better than those irrigated with canal water, due to their satisfying crop requirements for N, P and K. The heavy metal concentrations were very low, and did not influence crop quality. More studies are needed to provide a scientific package to be followed by farmers to minimize the risks of the long-term use of recycled wastewater in irrigation.

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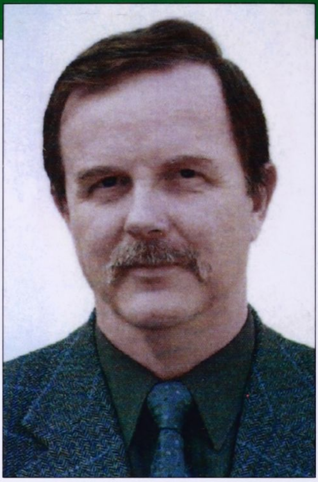

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